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(54) Title: GROWING MARINE FISH IN FRESHWATER

(57) Abstract: The invention relates to methods, compositions and kits for raising marine fish in freshwater. The methods involve adding at least one Polyvalent Cation Sensing Receptor (PVCR) modulator to the freshwater in an amount sufficient to increase expression and/or sensitivity of at least one PVCR; and adding feed for fish consumption of the freshwater, wherein the feed comprises an amount of NaCl sufficient to contribute to a significant increased level of the PVCR modulator in serum of the marine fish.

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GROWING MARINE FISH IN FRESHWATER

RELATED APPLICATION

This application is a continuation-in-part of Application No. 09/687,373, filed October 12, 2000. The entire teachings of the above application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Growing marine fish has been generally limited to costal regions or seawater tanks. However, many freshwater aquifers exist, for example, in the Midwest as potential environments for the raising of marine fish. Until now, attempts to grow marine fish in freshwater have been unsuccessful.

Growing marine fish in freshwater would provide an opportunity for non-costal areas to raise marine fish. The ability to grow marine fish in freshwater can provide fresh fish and economic growth to these areas.

Hence, a need exists to determine whether it is possible to adapt a marine fish to freshwater, and if so, understand the biological mechanisms that allow a marine fish to do so. In particular, a need exists to grow marine fish in freshwater.

SUMMARY OF THE INVENTION

The present invention relates to methods of growing marine fish in freshwater by increasing or maintaining expression of a receptor, referred to as the Polyvalent Cation Sensing Receptor (PVCR). The expression and/or sensitivity of the PVCR is modulated or maintained by subjecting the marine fish to at least one modulator of the PVCR. The marine fish are subjected to the modulator when it is added to the freshwater environment, and optionally, to the feed.

In one embodiment, the present invention is directed toward a method of growing marine fish in freshwater comprising adding at least one Polyvalent Cation Sensing Receptor (PVCR) modulator to freshwater in an amount sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR in one or more tissues; transferring the marine fish to the freshwater and adding feed for fish

consumption to the modified freshwater, wherein the feed contains an amount of NaCl sufficient to contribute to modulate or maintain levels of said PVCR modulator in serum of the marine fish. PVCR modulators useful in the present invention include a divalent cation, a trivalent cation, an aminoglycoside, an organic polycation, an amino acid, a Type I Calcimimetic, a Type II Calcimimetic, 1,25 dihydroxyvitamin D, a cytokine, and macrophage chemotatic peptide-1. The feed suitable in the methods of the present invention contains at least about 1% NaCl by weight and can optionally include a PVCR modulator.

The present invention also encompasses a method of transferring marine fish to freshwater comprising adding at least one Polyvalent Cation Sensing Receptor (PVCR) modulator to the freshwater in an amount sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR in one or more tissues, transferring the marine fish to the freshwater, adding feed for fish consumption to the modified freshwater, wherein the feed contains at least about 1% NaCl by weight. The PVCR modulator can be a PVCR agonist, a divalent cation, a trivalent cation, an aminoglycoside, an organic polycation or an amino acid.

In another embodiment, the present invention is directed toward a method of growing marine fish in freshwater comprising determining the level of at least one PVCR modulator in freshwater, adding said PVCR modulator to the freshwater in an amount sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR in one or more tissues, transferring the marine fish to the freshwater and adding feed for fish consumption to the modified freshwater, wherein the feed contains an amount of NaCl sufficient to modulate or maintain levels of said PVCR modulator in serum of the marine fish (calcium and magnesium). PVCR modulator that can be assessed. The PVCR modulator is added to freshwater such that the freshwater has between about 0.3 mM and about 10.0 mM calcium and between about 0.5 mM and about 10.0 mM magnesium prior to transferring marine fish.

The present invention is also directed to a method of growing marine fish in freshwater having between about 0.3 mM and about 10.0 mM of calcium and between about 0.5 mM and 10.0 mM of magnesium. The method comprises adding feed to the freshwater wherein the feed contains an amount of NaCl sufficient to

modulate or maintain levels of said PVCR modulator in serum of the marine fish, wherein modulated or maintained expression of at least one PVCR is modulated or maintained in one or more tissues.

In another embodiment, the present invention is directed toward a method of transferring marine fish to freshwater comprising transferring the marine fish to freshwater having magnesium and calcium in the freshwater in amounts sufficient to modulate or maintain the expression and/or sensitivity of at least one PVCR in one or more tissues and adding feed to the freshwater, wherein the feed contains at least about 1% NaCl by weight.

The present invention is also directed to a method of growing flounder in freshwater comprising transferring flounder to freshwater having at least one PVCR modulator in an amount sufficient to increase or maintain expression and/or sensitivity of at least one PVCR in one or more tissue and adding feed for fish consumption to the freshwater, wherein the feed contains an amount of NaCl sufficient to contribute to a significant increased level of said PVCR modulator in serum of the flounder. The pH of the freshwater should be greater than 7.0.

In another embodiment, the present invention is directed toward an aquatic mixture for providing an environment to transfer marine fish to freshwater, comprising at least one PVCR modulator. An aquatic mixture is a medium suitable for transfer of marine fish to freshwater during aquaculture.

The present invention is also directed to a kit for growing marine fish in freshwater comprising an aquatic mixture for providing an environment to grow the marine fish, wherein the aquatic mixture comprises at least one PVCR modulator; and an aquatic food composition containing a concentration of NaCl between about 10,000 mg/kg and about 100,000 mg/kg.

Surprisingly, it has been discovered that modulated or maintained expression and/or altering the sensitivity of the PVCR allows these marine fish to live and thrive in freshwater. Until the discovery of the present invention, the aquaculture industry was unable to transfer the marine fish to freshwater without subjecting the fish to stress, death and/or disease. Unlike this practice, carrying out the steps of the invention modulates or maintains the expression and/or alters the sensitivity of the

PVCR and allows for transfer of the marine fish to freshwater with minimal or no stress, death and/or disease, and unexpectedly, the fish grow. In fact, marine fish that grow in freshwater have a higher fat content, and a milder, less "fishy" taste.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are diagrams illustrating the partial nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of the PVCR of Cod.

Figures 2A and 2B are diagrams illustrating the partial nucleotide (SEQ ID NO:3) and amino acid (SEQ ID NO:4) sequences of the PVCR of Haddock.

Figure 3A and 3B are diagrams illustrating the partial nucleotide (SEQ ID NO:5) and amino acid (SEQ ID NO:6) sequences of the PVCR of Hake.

Figures 4A-B are diagrams illustrating the partial nucleotide (SEQ ID NO:7) and amino acid (SEQ ID NO:8) sequences of the PVCR of Halibut.

Figure 5A-B are diagrams illustrating the partial nucleotide (SEQ ID NO:9) and amino acid (SEQ ID NO:10) sequences of the PVCR of Mackerel.

Figures 6A-B are diagrams illustrating the partial nucleotide (SEQ ID NO:11) and amino acid (SEQ ID NO:12) sequences of the PVCR of Pollack.

Figure 7A-B are diagrams illustrating the partial nucleotide (SEQ ID NO:13) and amino acid (SEQ ID NO:14) sequences of the PVCR of Sea Bass.

Figures 8A-B are diagrams illustrating the partial nucleotide (SEQ ID NO:15) and amino acid (SEQ ID NO:16) sequences of the PVCR of Swordfish.

Figures 9A-B are diagrams illustrating the partial nucleotide (SEQ ID NO:17) and amino acid (SEQ ID NO:18) sequences of the PVCR of Tuna.

Figures 10A-C are diagrams illustrating the partial nucleotide (SEQ ID NO:19) and amino acid (SEQ ID NO:20) sequences of the PVCR of Winter Flounder.

Figure 11 is a diagram illustrating the partial nucleotide (SEQ ID NO: 21) and amino acid (SEQ ID NO: 22) sequences of PVCR of Summer Flounder.

Figures 12A-D are diagrams illustrating the alignment of the nucleic acids sequences for Cod (SEQ ID NO: 1), Haddock (SEQ ID NO: 3), Hake (SEQ ID NO: 5), Halibut (SEQ ID NO: 7), Mackerel (SEQ ID NO: 9), Pollock (SEQ ID NO: 11),

Sea Bass (SEQ ID NO: 13), Swordfish (SEQ ID NO: 15), Tuna (SEQ ID NO: 17), Winter Flounder (SEQ ID NO: 19).

Figures 13A-C are diagrams illustrating the alignment of the amino acids sequences for Cod (SEQ ID NO: 2), Haddock (SEQ ID NO: 4), Hake (SEQ ID NO: 6), Halibut (SEQ ID NO: 8), Mackerel (SEQ ID NO: 10), Pollock (SEQ ID NO: 12), Sea Bass (SEQ ID NO: 14), Swordfish (SEQ ID NO: 16), Tuna (SEQ ID NO: 18), Winter Flounder (SEQ ID NO: 20).

Figures 14A-B are diagrams illustrating the nucleic acid sequence of SKCaR (SEQ ID NO.: 23).

Figure 15 is a graphical representation illustrating the growth of summer flounder in freshwater that underwent APS Process I and grown in freshwater for a total of 51 days. Samples of body characteristics of flounders were obtained at (1) prior to placement in freshwater; (2) 20 days after placement in freshwater; (3) 30 days after placement in freshwater; and (4) 51 days after placement in freshwater. APS Process I is defined in Example 2.

Figure 16 is a graphical representation illustrating the growth of summer flounder in seawater for a total of 51 days. Samples of body characteristics of flounders were obtained at (1) prior to placement in seawater; (2) 20 days after placement in seawater; (3) 30 days after placement in seawater; and (4) 51 days after placement in seawater.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for growing or raising marine fish in freshwater. The methods involve modulating or maintaining expression and/or altering the sensitivity of a Polyvalent Cation Sensing Receptor (PVCR) (*e.g.*, at least one PVCR). The invention relates to modulating or maintaining expression of the PVCR that affects the fish's ability to adapt to freshwater.

In particular, the methods of the present invention include adding at least one PVCR modulator to the freshwater, and adding a specially made or modified feed to the freshwater for consumption by the fish. The feed contains a sufficient amount of sodium chloride (NaCl) (*e.g.*, between about 1% and about 10% by weight, or about

10,000 mg/kg to about 100,000 mg/kg) to modulate or maintain levels of the PVCR modulator in the serum. This amount of NaCl in the feed causes or induces the marine fish to drink more freshwater. Since the freshwater contains a PVCR modulator and the fish ingest increased amounts of it, the serum level of the PVCR modulator significantly increases in the fish, and causes increased or maintained PVCR expression and/or altered PVCR sensitivity. A "significant" increase is used herein to refer to a measurable rise in the level or quantity of PVCR or RVCR modulator as compared to a control or reference. Methods of measuring or detecting a significant increase in PVCR or PVCR modulator are disclosed herein and known to one skilled in the art.

The methods of the present invention pertain to adapting marine fish to freshwater. Marine fish are fish that live, at least for most of their adult lives, in seawater. Marine fish include, for example, Cod, Haddock, Hake, Halibut, Mackerel, Pollock, Sea Bass, Swordfish, Tuna, Winter Flounder, and Summer Flounder. The marine fish are adapted to freshwater having a PVCR modulator.

The term "marine fish" is understood by one of skill in the art. The term, "freshwater," means water that comes from, for example, a stream, river, ponds, public water supply, or from other non-marine sources having, for example, the following ionic composition: less than about 2 mM of magnesium, calcium and NaCl. The phrases "modified freshwater," "freshwater as modified by the addition of a PVCR" and "PVCR modulator environment" refer to freshwater to which at least one PVCR modulator has been added, as described herein.

The PVCR modulator is added to the freshwater in sufficient amounts to modulate or maintain expression or alter the sensitivity of at least one PVCR. A PVCR has been isolated from various tissue of several types of marine fish using molecular biological techniques. For example, nucleic acid was isolated from tissue samples from various species of marine fish including Cod, Haddock, Hake, Halibut, Mackerel, Pollock, Sea Bass, Swordfish, Tuna, Winter Flounder and Summer Flounder. The nucleic acid was amplified using Polymerase Chain Reaction (PCR) methodology. The amplified DNA was purified, subcloned into vectors, and their sequences were determined, as described in Example 4.

The PVCR, which is located in various tissues (*e.g.*, gill, skin, intestine, kidney, urinary bladder, brain or muscle) of the marine fish, senses alterations in PVCR modulators including various ions (*e.g.*, divalent cations), for example, in the surrounding water, in their serum or in the luminal contents of tubules inside the body, such as kidney, urinary bladder, or intestine. The ability to sense PVCR modulators results in a modulation or a maintenance in the expression of PVCR, thereby allowing the fish to better adapt to freshwater. Modulated or maintained expression of the PVCR can occur, for example, in one or more tissues. As used herein, the "sensitivity" of the PVCR refers to alteration of PVCR expression in response to a change in the concentration of PVCR modulators. PVCR expression can be assessed by measuring or detecting PVCR polypeptide or nucleic acid molecules in a sample by standard methods.

A "PVCR modulator" is defined herein to mean a compound which modulates expression of the PVCR, or modulates the sensitivity or responsiveness of the PVCR, or maintains an already increased PVCR expression level in one or more tissues. Such compounds include, but are not limited to, PVCR agonists (*e.g.*, inorganic polycations, organic polycations and amino acids), Type II calcimimetics, and compounds that indirectly alter PVCR expression (*e.g.*, 1,25 dihydroxyvitamin D in concentrations of about 3,000-10,000 International Units/kg feed), cytokines such as Interleukin Beta, and Macrophage Chemotatic Peptide-1 (MCP-1)). Examples of Type II calcimimetics, which modulate expression and/or sensitivity of the PVCR, are, for example, NPS-R-467 and NPS-R-568 from NPS Pharmaceutical Inc., (Salt Lake, Utah, Patent Nos. 5,962,314; 5,763,569; 5,858,684; 5,981,599; 6,001,884) which can be administered in concentrations of between about 0.1 μM and about 100 μM feed or water. See Nemeth, E.F. *et al., PNAS* 95: 4040-4045 (1998).

Examples of inorganic polycations are divalent cations including calcium at a concentration between about 0.3 and about 10.0 mM and magnesium at a concentration between about 0.5 and about 10.0 mM; and trivalent cations including, but not limited to, gadolinium (Gd3+) at a concentration between about 1 and about 500 μM.

Organic polycations include, but are not limited to, aminoglycosides such as neomycin or gentamicin in concentrations of between about 1 and about 8 gm/kg feed as well as organic polycations including polyamines (*e.g.*, polyarginine, polylysine, polyhistidine, polyornithine, spermine, cadaverine, putricine, copolymers of poly arginine/histidine, poly lysine/arginine in concentrations of between about 10 μM and 10 mM feed). See Brown, E.M. *et al., Endocrinology* 128: 3047-3054 (1991); Quinn, S.J. *et al., Am. J. Physiol.* 273: C1315-1323 (1997).

Additionally, PVCR agonists include amino acids such as L-Tryptophan, L-Tyrosine, L-Phenylalanine, L-Alanine, L-Serine, L-Arginine, L-Histidine, L-Leucine, L-Isoleucine, and L-Cystine at concentrations of between about 1 and about 10 gm/kg feed. See Conigrave, A.D., *et al., PNAS* 97: 4814-4819 (2000). The molar concentrations refer to free or ionized concentrations of the PVCR modulator in the freshwater, and do not include amounts of bound PVCR modulator (*e.g.*, PVCR modulator bound to negatively charged particles including glass, proteins, or plastic surfaces). Any combination of these modulators can be added to the water or to the feed (in addition to the NaCl, as described herein), so long as the combination modulates or maintains expression and/or sensitivity of at least one PVCR.

The PVCR modulator can be administered to the fish in a number of ways. The invention encompasses administration of the PVCR in any way that is sufficient to modulate or maintain the expression and/or alter the sensitivity of the PVCR. In one embodiment, the PVCR modulator is simply added to the freshwater, as described herein. PVCR modulators that are added to the water increase or maintain or decrease expression and/or alter the sensitivity of the PVCR on the skin and gills of the fish, and can be ingested by the fish, in particular, when fish are fed feed having between about 1% and about 10% NaCl (*e.g.*, in concentrations between about 1 and about 10 gm/100 gm feed). In addition to adding NaCl to the feed, the PVCR modulator can also be added to the feed. Amounts and types of PVCR modulators added to the feed are also described herein. Other embodiments include subjecting the fish to the PVCR modulator by "dipping" the fish in the modulator, *e.g.*, organic polycations. The organic polycations can be formulated in such a way

as to allow the polycations to adhere to the skin and gills of the fish, in sufficient amounts to increase or maintain expression of the PVCR.

The invention also embodies assessing the amounts of existing PVCR modulator in the freshwater environment and in the serum of fish. PVCR modulators are assessed or measured using methods known in the art. After assessment, the PVCR modulator is added to the water to bring the concentration up to an amount sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR, or sufficient to bring the concentrations of the PVCR modulator within the stated ranges. For example, an aquifer assessed at having only 0.2 mM of calcium needs additional calcium to bring the concentration up to between about 0.3 mM and between about 10.0 mM.

In a preferred embodiment, the present invention is practiced by adding a combination of two PVCR agonists to the freshwater. In particular, calcium and magnesium are added to the freshwater to bring the concentrations of each to between about 0.3 mM and about 10.0 mM of calcium, and between about 0.5 mM and about 10.0 mM of magnesium. In addition to adding calcium and magnesium to the water, these ranges of ion concentrations can be achieved by providing a brackish water (*e.g.*, diluted seawater) environment for the fish.

Calcium and magnesium can come from a variety of sources, that when added to the water, the calcium and/or magnesium levels modulate or maintain expression of the PVCR, and/or are within the stated ranges. Sources of calcium and magnesium can be a mixture of a variety of compounds, or each can come from a substantially uniform or pure compound. Sources of calcium include, for example, $Ca(CO_3)_2$, $CaCl_2$, and $CaSO_4$ and sources of magnesium include, for example, $MgCl_2$, $MgSO_4$, $MgBr_2$, and $MgCO_3$.

In one embodiment, the invention includes intermittent (*e.g.*, interrupted) as well as continuous (*e.g.*, non-interrupted) exposure to freshwater having at least one PVCR modulator, while on the NaCl diet. Intermittent exposure to the PVCR can occur so long as the PVCR expression and/or altered sensitivity remains modulated or maintained. Continuous maintenance in or exposure to freshwater having at least one PVCR modulator is shown in Example 2.

The marine fish are transferred from seawater. The term, "seawater," means water that comes from the sea, or water which has been formulated to simulate the chemical and mineral composition of water from the sea. The major elemental composition of the prepared seawater preferably falls substantially within the range of the major elemental composition of the natural seawater (*e.g.*, having the following ionic composition: greater than 30 mM of magnesium, greater than about 6 mM of calcium, and greater than about 300 mM NaCl). Methods of preparing artificial seawater are known in the art and are described in, for instance, U.S. Pat. No. 5,351,651.

In one embodiment, the marine fish are treated by the methods of the present invention by subjecting the fish to a gradual or step-wise decrease in salinity for a period of time prior to transfer to freshwater, while being fed a NaCl diet. Salinity refers to the ionic concentrations (*e.g.*, calcium, magnesium and sodium) in water. The fish are maintained in a decreasing salinity environment for a sufficient period of time to modulate or maintain expression and/or sensitivity of at least one PVCR. Factors that can influence the length of time to maintain the fish in a decreased salinity prior to transfer to freshwater include, but are not limited to, size of the fish, level of PVCR expression or sensitivity, if any, prior to addition of the PVCR modulator to the freshwater, the fish's ability to excrete the PVCR modulator and ions, and the fish's surface to volume ratio. Therefore, the length of time the fish is maintained can range between about 5 days and about 60 days, and preferably, between about 10 days and about 25 days.

The ionic concentrations of seawater are decreased by between about 10% and about 90%, and preferably, between about 25% and about 50%. Combinations of decreasing salinity and various lengths of exposure to the salinity are encompassed by the invention. For example, as described in Example 2, fish were adapted to 50% seawater (50% salinity of seawater) for 10 days, and then adapted to 25% seawater (25% salinity of seawater) for 15 days, prior to transfer to freshwater. "Adapted" as used herein, refers to a successful transition to the altered aquatic environment. After maintenance in water having decreasing salinity, as compared to seawater, the marine fish are then placed into freshwater having a PVCR modulator,

as described herein. The fish can remain and grow in freshwater, modified by the addition of PVCR modulators, indefinitely, so long as there is modulated or maintained expression and/or sensitivity of the PVCR (*e.g.*, maintained in modified freshwater and fed an NaCl diet).

The invention further includes adding feed to the freshwater. The frequency and amount of feed that fish are fed, are taught in the art. Generally, the fish are fed 1-3 times a day, totaling about 0.25-0.5% body weight/day. The feed has enough NaCl to contribute to a modulated or maintained level of the PVCR modulator in the serum of the marine fish. Specifically, the presence of sufficient amounts of NaCl in the feed causes the marine fish to drink more water from the surrounding environment. Although NaCl decreases PVCR sensitivity, the ingestion of freshwater having at least one PVCR modulator causes an overall rise in the serum level of PVCR modulator. The increase in serum levels of PVCR modulator results in a modulation in expression of PVCR.

In another embodiment, the present invention is directed toward an aquatic mixture for providing an environment to transfer marine fish to freshwater, comprising at least one PVCR modulator. An "aquatic mixture" is defined herein to mean a composition that provides a suitable environment for the successful transfer of marine fish to freshwater by the methods of the present invention. The aquatic mixture can be premixed for immediate use in the methods of the present invention. Alternatively, the aquatic mixture can require reconstitution with water. The aquatic mixture when reconstituted yields a solution comprising about 0.3-10 mM Ca^{2+} and about 0.5-10 mM Mg^{2+}. The aquatic mixture can optionally include an amino acid in an amount between about 1 gm/ kg and about 10 gm/kg.

The present invention also relates to an aquatic food composition. An "aquatic food composition" refers to fish feed, as described herein. An aquatic food composition or feed suitable for use in the present invention contains between about 1%-10% of NaCl by weight, or between about 10,000 mg NaCl/kg of feed and about 100,000 mg NaCl/kg of feed (*e.g.*, 12,000 mg/kg). The feed is referred to herein as a "NaCl diet." The NaCl can be combined with other sodium salts to confer the desired effect of modulating or maintaining PVCR expression, altering PVCR

sensitivity and/or inducing the fish to drink more. Hence, as used herein, the term NaCl, includes a substantially pure compound, mixtures of NaCl with other sources of sodium, or other sources of sodium. The feed can further include a PVCR modulator, and in particular a PVCR agonist such as an amino acid. In one embodiment, the feed has between about 1% and about 10% NaCl by weight and an amino acid such as tryptophan in an amount between about 1 and about 10 gm/kg. This embodiment is referred to herein as "APS Process II," which is further defined in Example 2.

The feed can be made in a number of ways, so long as the proper concentration of NaCl is present. The feed can be made, for example, by reformulating the feed, or by allowing the feed to absorb a solution having the NaCl and optionally, adding a PVCR modulator. Additionally, a top dressing can be added for palatability. Example 3 describes in detail one way to make the feed. Alternate methods of preparing fish feed are know to those of skill in the relevant art.

Another embodiment of the present invention includes feeding marine fish feed having between 1% and 10% NaCl by weight when the fish are maintained in a freshwater environment having between about 0.3 and about 10.0 mM of calcium, and between about 0.5 mM and about 10.0 mM of magnesium. When this embodiment of the present invention is carried out, the levels of calcium, magnesium and/or sodium in the serum of the marine fish is increased, as compared to PVCR expression and/or sensitivity seen in freshwater fish.

In another embodiment, the fish, while in water having decrease salinity, as compared to seawater, or while in the freshwater having the PVCR modulator, are also exposed to a photoperiod. A photoperiod refers to exposing the fish to light (*e.g.*, sunlight, incandescent light or fluorescent light). Preferably, the photoperiod is substantially continuous, or occurs long enough to increase growth. The photoperiod can occur for at least about 12 hours within a 24 hour interval, or for longer periods such as about 14, 16, 18, 20, 22 or preferably, about 24 hours.

The methods of the present invention modulate or maintain the expression and/or sensitivity of the PVCR in marine fish which results in reduced osmotic

stress and in reduced mortality. Marine fish cultured in freshwater by methods of the present invention consume feed and exhibit growth. In contrast, marine fish that are not cultured in freshwater by methods of the present invention experience osmotic stress, reduced or no food consumption, and eventually death. The osmotic stress results from differences in the osmotic pressure between the surrounding environment and body compartments of the fish. This disturbs the homeostatic equilibrium of the fish and results in decreased growth, reproductive failure and reduced resistance to disease. The fish that have undergone the steps of the present invention do not experience a significant amount of osmotic stress. As a result, the fish are able to grow. Surprisingly, as described and exemplified herein, marine fish adapted by the present invention grow almost as well as marine fish maintained in seawater (*e.g.*, 53% increased growth in fish subjected to the present invention for 37 days, as compared to 60% increased growth of fish maintained in seawater for 37 days). Additionally, marine fish cultured in freshwater by methods of the present invention exhibit a survival rate that is significantly greater than the rate for marine fish that are transferred directly to freshwater and not subjected to the steps of the present invention (*e.g.*, between about 60% and about 100%). See Figures 15 and 16.

The methods of the present invention also decrease the incidence of disease among the marine fish transferred to freshwater. Because the fish treated with the methods of the present invention experience less stress upon transfer to freshwater, their immune functions are stronger, and they are less susceptible to parasitic, viral, bacterial and fungal diseases. Thus, marine fish cultured by methods of the present invention are healthier.

Methods Assessment of the PVCR

The present invention includes methods of detecting the level of the PVCR to determine whether fish are ready for transfer from seawater to freshwater. Methods that measure PVCR levels include several suitable assays. Suitable assays encompass immunological methods, such as FACS analysis, radioimmunoassay, flow cytometry, enzyme-linked immunosorbent assays (ELISA) and

chemiluminescence assays. Any method known now or developed later can be used for measuring PVCR expression.

Antibodies reactive with the PVCR or portions thereof can be used. In a preferred embodiment, the antibodies specifically bind with the PVCR or a portion thereof. The antibodies can be polyclonal or monoclonal, and the term antibody is intended to encompass polyclonal and monoclonal antibodies, and functional fragments thereof. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production.

In several of the preferred embodiments, immunological techniques detect PVCR levels by means of an anti-PVCR antibody (*i.e.*, one or more antibodies). The term "anti-PVCR" antibody includes monoclonal and/or polyclonal antibodies, and mixtures thereof.

Anti-PVCR antibodies can be raised against appropriate immunogens, such as isolated and/or recombinant PVCR or portion thereof (including synthetic molecules, such as synthetic peptides). In one embodiment, antibodies are raised against an isolated and/or recombinant PVCR or portion thereof (*e.g.*, a peptide) or against a host cell which expresses recombinant PVC R. In addition, cells expressing recombinant PVCR, such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor.

Any suitable technique can prepare the immunizing antigen and produce polyclonal or monoclonal antibodies. The art contains a variety of these methods (see *e.g.*, Kohler *et al.*, *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein *et al.*, *Nature*, 266: 550-552 (1977); Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); Current Protocols In Molecular Biology, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, fusing antibody producing cells with a suitable immortal or myeloma cell line, such as SP2/0, can produce a hybridoma. For example, animals immunized with the antigen of interest provide the antibody producing cell, preferably cells

from the spleen or lymph nodes. Selective culture conditions isolate antibody producing hybridoma cells while limiting dilution techniques produce them. Researchers can use suitable assays such as ELISA to select antibody producing cells with the desired specificity.

Other suitable methods can produce or isolate antibodies of the requisite specificity. Examples of other methods include selecting recombinant antibody from a library or relying upon immunization of animals such as mice.

According to the method, an assay can determine the level of PVCR in a biological sample. In determining the amounts of PVCR, an assay includes combining the sample to be tested with an antibody having specificity for the PVCR, under conditions suitable for formation of a complex between antibody and the PVCR, and detecting or measuring (directly or indirectly) the formation of a complex. The sample can be obtained directly or indirectly, and can be prepared by a method suitable for the particular sample and assay format selected.

In particular, tissue samples, *e.g.*, gill tissue samples, can be taken from fish after they are anaesthetized with MS-222. The tissue samples are fixed by immersion in 2% paraformaldehyde in appropriate Ringers solution corresponding to the osmolality of the fish, washed in Ringers, then frozen in an embedding compound, *e.g.*, O.C.T.™ (Miles, Inc., Elkahart, Indiana, USA) using methylbutane cooled with liquid nitrogen. After cutting 8-10μ tissue sections with a cryostat, individual sections are subjected to various staining protocols. For example, sections are: 1) blocked with goat serum or serum obtained from the same species of fish, 2) incubated with rabbit anti-CaR or anti-PVCR antiserum, and 3) washed and incubated with peroxidase-conjugated affinity-purified goat antirabbit antiserum. The locations of the bound peroxidase-conjugated goat antirabbit antiserum are then visualized by development of a rose-colored aminoethylcarbazole reaction product. Individual sections are mounted, viewed and photographed by standard light microscopy techniques. One anti-CaR antiserum used to detect fish PVCR protein is raised in rabbits using a 23-mer peptide corresponding to amino acids numbers 214-236 localized in the extracellular domain of the RaKCaR protein (Riccardi *et al.*, P.N.A.S. 92:131-135 (1995); accession number NP 058692). The sequence of

the 23-mer peptide is: ADDDYGRPGIEKFREEAEERDIC (SEQ ID NO.: 24) A small peptide with the sequence DDYGRPGIEKFREEAEERDICI (SEQ ID NO.: 25) or ARSRNSADGRSGDDLPC (SEQ ID NO.: 26) can also be used to make antisera containing antibodies to PVCRs. Such antibodies can be monoclonal, polyclonal or chimeric.

Suitable labels can be detected directly, such as radioactive, fluorescent or chemiluminescent labels. They can also be indirectly detected using labels such as enzyme labels and other antigenic or specific binding partners like biotin. Examples of such labels include fluorescent labels such as fluorescein, rhodamine, chemiluminescent labels such as luciferase, radioisotope labels such as ^{32}P, ^{125}I, ^{131}I, enzyme labels such as horseradish peroxidase, and alkaline phosphatase, β-galactosidase, biotin, avidin, spin labels and the like. The detection of antibodies in a complex can also be done immunologically with a second antibody which is then detected (*e.g.*, by means of a label). Conventional methods or other suitable methods can directly or indirectly label an antibody.

In performing the method, the levels of the PVCR are distinct from the control. Varied levels or the presence of PVCR expression, as compared to a control, indicate that the fish or the population of fish from which a statistically significant amount of fish were tested, are ready for transfer to freshwater. A control refers to a level of PVCR, if any, from a fish that is not subjected to the steps of the present invention, *e.g.*, not subjected to freshwater having a PVCR modulator and/or not fed a NaCl diet.

The PVCRs can also be assayed by Northern blot analysis of mRNA from tissue samples. Northern blot analysis from various shark tissues has revealed that the highest degree of PVCRs expression is in gill tissue, followed by the kidney and the rectal gland. There appear to be at least three distinct mRNA species of about 7 kb, 4.2 kb and 2.6 kb. For example, the PVCRs can also be assayed by hybridization, *e.g.*, by hybridizing one of the PVCR sequences provided herein (*e.g.*, SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 or 23), its complement or an oligonucleotide derived from one of the sequences, to a mRNA purified from tissue sample from a fish. Such a hybridization sequence can have a detectable label, *e.g.*,

radioactive, fluorescent, etc., attached, to allow the detection of hybridization product. Methods for hybridization are well known, and such methods are provided in U.S. Pat. No. 5,837,490, by Jacobs *et al.*, the entire teachings of which are herein incorporated by reference in their entirety. The design of the oligonucleotide probe should preferably follow these parameters: (a) it should be designed to an area of the sequence which has the fewest ambiguous bases ("N's"), if any, and (b) it should be designed to have a T_m of approx. 80°C (assuming 2°C for each A or T and 4 degrees for each G or C).

Stringency conditions for hybridization refers to conditions of temperature and buffer composition which permit hybridization of a first nucleic acid sequence to a second nucleic acid sequence, wherein the conditions determine the degree of identity between those sequences which hybridize to each other. Therefore, "high stringency conditions" are those conditions wherein only nucleic acid sequences which are very similar to each other will hybridize. The sequences can be less similar to each other if they hybridize under moderate stringency conditions. Still less similarity is needed for two sequences to hybridize under low stringency conditions. By varying the hybridization conditions from a stringency level at which no hybridization occurs, to a level at which hybridization is first observed, conditions can be determined at which a given sequence will hybridize to those sequences that are most similar to it. The precise conditions determining the stringency of a particular hybridization include not only the ionic strength, temperature, and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequences, their base composition, the percent of mismatched base pairs between the two sequences, and the frequency of occurrence of subsets of the sequences (*e.g.*, small stretches of repeats) within other non-identical sequences. Washing is the step in which conditions are set so as to determine a minimum level of similarity between the sequences hybridizing with each other. Generally, from the lowest temperature at which only homologous hybridization occurs, a 1% mismatch between two sequences results in a 1°C decrease in the melting temperature (T_m) for any chosen SSC concentration. Generally, a doubling of the concentration of SSC results in an

increase in the T_m of about 17°C. Using these guidelines, the washing temperature can be determined empirically, depending on the level of mismatch sought. Hybridization and wash conditions are explained in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., John Wiley & Sons, Inc., 1995, with supplemental updates) on pages 2.10.1 to 2.10.16, and 6.3.1 to 6.3.6.

High stringency conditions can employ hybridization at either (1) 1x SSC (10x SSC = 3 M NaCl, 0.3 M Na_3-citrate·$2H_2O$ (88 g/liter), pH to 7.0 with 1 M HCl), 1% SDS (sodium dodecyl sulfate), 0.1 - 2 mg/ml denatured calf thymus DNA at 65°C, (2) 1x SSC, 50% formamide, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (3) 1% bovine serum albumen (fraction V), 1 mM Na_2·EDTA, 0.5 M $NaHPO_4$ (pH 7.2) (1 M $NaHPO_4$ = 134 g Na_2HPO_4·$7H_2O$, 4 ml 85% H_3PO_4 per liter), 7% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 65°C, (4) 50% formamide, 5x SSC, 0.02 M Tris-HCl (pH 7.6), 1x Denhardt's solution (100x = 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (fraction V), water to 500 ml), 10% dextran sulfate, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (5) 5x SSC, 5x Denhardt's solution, 1% SDS, 100 µg/ml denatured calf thymus DNA at 65°C, or (6) 5x SSC, 5x Denhardt's solution, 50% formamide, 1% SDS, 100 µg/ml denatured calf thymus DNA at 42°C, with high stringency washes of either (1) 0.3 - 0.1x SSC, 0.1% SDS at 65°C, or (2) 1 mM Na_2EDTA, 40 mM $NaHPO_4$ (pH 7.2), 1% SDS at 65°C. The above conditions are intended to be used for DNA-DNA hybrids of 50 base pairs or longer. Where the hybrid is believed to be less than 18 base pairs in length, the hybridization and wash temperatures should be 5 - 10°C below that of the calculated T_m of the hybrid, where T_m in °C = (2 x the number of A and T bases) + (4 x the number of G and C bases). For hybrids believed to be about 18 to about 49 base pairs in length, the T_m in °C = (81.5°C + 16.6($\log_{10}$M) + 0.41(% G + C) - 0.61 (% formamide) - 500/L), where "M" is the molarity of monovalent cations (*e.g.*, Na^+), and "L" is the length of the hybrid in base pairs.

Moderate stringency conditions can employ hybridization at either (1) 4x SSC, (10x SSC = 3 M NaCl, 0.3 M Na_3-citrate·$2H_2O$ (88 g/liter), pH to 7.0 with 1 M HCl), 1% SDS (sodium dodecyl sulfate), 0.1 - 2 mg/ml denatured calf thymus DNA

at 65°C, (2) 4x SSC, 50% formamide, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (3) 1% bovine serum albumen (fraction V), 1 mM Na_2·EDTA, 0.5 M $NaHPO_4$ (pH 7.2) (1 M $NaHPO_4$ = 134 g $Na_2HPO_4 \cdot 7H_2O$, 4 ml 85% H_3PO_4 per liter), 7% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 65°C, (4) 50% formamide, 5x SSC, 0.02 M Tris-HCl (pH 7.6), 1x Denhardt's solution (100x = 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (fraction V), water to 500 ml), 10% dextran sulfate, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (5) 5x SSC, 5x Denhardt's solution, 1% SDS, 100 µg/ml denatured calf thymus DNA at 65°C, or (6) 5x SSC, 5x Denhardt's solution, 50% formamide, 1% SDS, 100 µg/ml denatured calf thymus DNA at 42°C, with moderate stringency washes of 1x SSC, 0.1% SDS at 65°C. The above conditions are intended to be used for DNA-DNA hybrids of 50 base pairs or longer. Where the hybrid is believed to be less than 18 base pairs in length, the hybridization and wash temperatures should be 5 - 10°C below that of the calculated T_m of the hybrid, where T_m in °C = (2 x the number of A and T bases) + (4 x the number of G and C bases). For hybrids believed to be about 18 to about 49 base pairs in length, the T_m in °C = (81.5°C + 16.6($\log_{10}$M) + 0.41(% G + C) - 0.61 (% formamide) - 500/L), where "M" is the molarity of monovalent cations (*e.g.*, Na^+), and "L" is the length of the hybrid in base pairs.

Low stringency conditions can employ hybridization at either (1) 4x SSC, (10x SSC = 3 M NaCl, 0.3 M Na_3-citrate·$2H_2O$ (88 g/liter), pH to 7.0 with 1 M HCl), 1% SDS (sodium dodecyl sulfate), 0.1 - 2 mg/ml denatured calf thymus DNA at 50°C, (2) 6x SSC, 50% formamide, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 40°C, (3) 1% bovine serum albumen (fraction V), 1 mM Na_2·EDTA, 0.5 M $NaHPO_4$ (pH 7.2) (1 M $NaHPO_4$ = 134 g $Na_2HPO_4 \cdot 7H_2O$, 4 ml 85% H_3PO_4 per liter), 7% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 50°C, (4) 50% formamide, 5x SSC, 0.02 M Tris-HCl (pH 7.6), 1x Denhardt's solution (100x = 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (fraction V), water to 500 ml), 10% dextran sulfate, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 40°C, (5) 5x SSC, 5x Denhardt's solution, 1% SDS, 100 µg/ml denatured calf thymus DNA at 50°C, or (6) 5x SSC, 5x Denhardt's solution, 50%

formamide, 1% SDS, 100 μg/ml denatured calf thymus DNA at 40°C, with low stringency washes of either 2x SSC, 0.1% SDS at 50°C, or (2) 0.5% bovine serum albumin (fraction V), 1 mM Na_2EDTA, 40 mM $NaHPO_4$ (pH 7.2), 5% SDS. The above conditions are intended to be used for DNA-DNA hybrids of 50 base pairs or longer. Where the hybrid is believed to be less than 18 base pairs in length, the hybridization and wash temperatures should be 5 - 10°C below that of the calculated T_m of the hybrid, where T_m in °C = (2 x the number of A and T bases) + (4 x the number of G and C bases). For hybrids believed to be about 18 to about 49 base pairs in length, the T_m in °C = (81.5°C + 16.6($\log_{10}$M) + 0.41(% G + C) - 0.61 (% formamide) - 500/L), where "M" is the molarity of monovalent cations (*e.g.*, Na^+), and "L" is the length of the hybrid in base pairs.

Hence, the present invention includes kits for the detection of the PVCR or the quantification of the PVCR having either antibodies specific for the PVCR protein or a portion thereof, or a nucleic acid sequence that can hybridize to the nucleic acid of the PVCR.

Alterations in the expression or sensitivity of PVCRs could also be accomplished by introduction of a suitable transgene. Suitable transgenes would include either the PVCR gene itself or modifier genes that would directly or indirectly influence PVCR gene expression. Methods for successful introduction, selection and expression of the transgene in fish oocytes, embryos and adults are described in Chen, TT *et al.*, Transgenic Fish, *Trends in Biotechnology* 8:209-215 (1990).

The present invention is further and more specifically illustrated by the following Examples, which are not intended to be limiting in any way.

EXEMPLIFICATION

Example 1. Polyvalent cation-sensing receptors (PVCRs) serve as salinity sensors in fish.

Polyvalent cation-sensing receptors (PVCRs) serve as salinity sensors in fish. These receptors are localized to the apical membranes of various cells within the fish's body (*e.g.*, in the gills, intestine, kidney) that are known to be responsible for

osmoregulation. A full-length cation receptor (CaR) from the dogfish shark has been expressed in human HEK cells. This receptor was shown to respond to alterations in ionic compositions of NaCl, Ca^{2+} and Mg^{2+} in extracellular fluid bathing the HEK cells. The ionic concentrations responded to encompassed the range which includes the transition from freshwater to seawater. Expression of PVCR mRNA is also modulated in fish after their transfer from freshwater to seawater, and is modulated by PVCR agonists.

Using nucleic acid amplification with degenerate primers, partial genomic clones of PVCRs have also been isolated from other fish species, including Cod (Figures 1A-B), Haddock (Figures 2A-B), Hake (Figures 3A-B), Halibut (Figures 4A-B), Mackerel (Figures 5A-B), Pollock (Figures 6A-B), Sea Bass (Figures 7A-B), Swordfish (Figures 8A-B), Tuna (Figures 9A-B), Winter Flounder (Figures 10A-10C) and Summer Flounder (Figure 11). The degenerate oligonucleotide primers used for isolating these clones, except for Winter Flounder, were 5'-TGT CKT GGA CGG AGC CCT TYG GRA TCG C-3' (SEQ ID NO:27) and 5'-GGC KGG RAT GAA RGA KAT CCA RAC RAT GAA G-3' (SEQ ID NO:28), where K is T or G, Y is C or T, and R is A or G. The degenerate oligos were generated by standard methodologies (Preston, G.M., 1993, "Polymerase chain reaction with degenerate oligonucleotide primers to clone gene family members," in: Methods in Mol. Biol., vol. 58, ed. A. Harwood, Humana Press, pp. 303-312). Nucleic acids from these species were amplified, purified by agarose gel electrophoresis, ligated into an appropriate plasmid vector (Novagen's pT7 Blue or Promega's pGEM-T) and transformed into an appropriate bacterial host strain (Novagens' Nova Blue Competent Cells or Promega's JM 109 competent cells). The plasmids and inserts were purified from the host cells, and sequenced. Figures 13A-C shows the deduced amino acid sequences and alignment for the PVCRs from Cod, Haddock, Hake, Halibut, Mackerel, Pollock, Sea Bass, Swordfish, Tuna and Winter Flounder.

A winter flounder lambda ZAP cDNA library was manufactured using standard commercially available reagents with cDNA synthesized from poly A+ RNA isolated from winter flounder urinary bladder tissue as described and published in Siner *et al. Am. J. Physiol.* 270:C372-C381, 1996. The winter flounder urinary

bladder cDNA library was plated and resulting phage plaques screened using a ^{32}P-labeled shark kidney calcium receptor cDNA probe under intermediate stringency conditions (0.5X SSC, 0.1% SDS, 50°C). Individual positive plaques were identified by autoradiography, isolated and rescued using phagemid infections to transfer cDNA to KS Bluescript vector. The nucleotide (nt) sequence, Figure 10A, (SEQ ID NO: 19) of the winter flounder PVCR clone was obtained using commercially available automated sequencing service that performs nucleotide sequencing using the dideoxy chain termination technique. The deduced amino acid sequence (SEQ ID NO: 20) is shown in Figures 10B and 10C. The winter flounder PVCR nucleotide sequence was compared to others aquatic PVCR using commercially available nucleotide and protein database services including GENBANK and SWISS PIR.

Example 2: Growth of Marine Fish in Freshwater Using the Methods of the Present Invention

Methods:

The following examples refer to APS Process I and APS Process II throughout. APS stands for "AquaBio Products Sciences®, L.L.C." APS Process I is also referred to herein as "SUPERSMOLT ™ I Process" or "Process I." An "APS Process I" fish or smolt refers to a fish or smolt that has undergone the steps of APS Process I. An APS Process I smolt is also referred to as a "SUPERSMOLT ™ I" or a "Process I " smolt. Likewise, APS Process II is also referred to herein as "SUPERSMOLT ™ II Process" or "Process II." An "APS Process II" fish or smolt refers to a fish or smolt that has undergone the steps of APS Process II. An APS Process II smolt is also referred to as a "SUPERSMOLT ™ II" or a "Process II " smolt.

APS Process I: Marine fish are exposed to or maintained in freshwater containing 0.3-10.0 mM calcium and 0.5-10.0 mM magnesium ions. This water is prepared by addition of calcium carbonate and/or calcium chloride and magnesium chloride to the freshwater. Fish are fed with feed pellets containing 1-7% (weight/weight) NaCl. See Example 3 for further details regarding the feed. Fish

are exposed to or maintained in this regimen of water mixture and feed for a total of 30-45 days, using standard hatchery care techniques. Water temperatures vary between 10-16°C. Fish are exposed to a constant photoperiod for the duration of APS Process I. A fluorescent light is used for the photoperiod.

APS Process II: Marine fish are exposed to or maintained in freshwater containing 0.3-10.0 mM calcium and 0.5-10.0 mM magnesium ions. This water is prepared by addition of calcium carbonate and/or calcium chloride and magnesium chloride to the freshwater. Fish are fed with feed pellets containing 1-7% (weight/weight) NaCl and either 2 gm or 4 gm of L-Tryptophan per kg of feed. See Example 3 for further details regarding the feed. Fish are exposed to or maintained in this regimen of water mixture and feed for a total of 30-45 days using standard hatchery care techniques. Water temperatures vary between 10-16°C. Fish are exposed to a constant photoperiod for the duration of APS Process II. A fluorescent light is used for the photoperiod.

Summer Flounders of various weights that were all derived from a single homogenous stock of farm raised animals (Great Bay AquaFarms Portsmouth, NH) were transported and placed in artificial seawater (Crystal Sea) within the APS laboratory. These were divided into two groups (n=13) and one maintain in seawater (Seawater Control) for a total of 81 days and fed a standard flounder diet (Corey Feeds, New Brunswick, Canada). The other (Freshwater) was adapted to APS Process I conditions over 30 days consisting of 5 mM Ca^{2+}, 8mM Mg^{2+} concentrations in the water and a 1.2% NaCl supplemented diet of 70% standard flounder feed (Corey Feeds, New Brunswick, Canada) and 30% ground squid. These flounder were then maintained in APS Process I conditions for a total of 51 days and their growth compared to that exhibited by matched paired summer flounder maintain in seawater.

Flounders were adapted to the APS Process I by the following 30 day schedule:

1. Maintenance in seawater for 5 days.
2. Reduce water salinity to 50% seawater for 10 days.
3. Reduce water salinity to 25% seawater for 15 days.

4. Place fish in APS Process I water (5 mM Ca^{2+}, 8mM Mg^{2+} concentrations in the freshwater, pH 7.6-8.0)

Fish were individually tagged using colored elastomer tags their change in weight was determined at specific time points during the 51 day experimental interval.

The feed conversion ratio or FCR is obtained by dividing the body weight gained by a group of fish into the amount of food fed to the group of fish. The more efficient the conversion of food into body weight growth by fish, the smaller the FCR (small amount of food/large weight gain of fish). A very small FCR number (less than 1) encompasses a highly efficient conversion of food into body weight growth which is the goal of aquaculture. By contrast, a large FCR means an inefficient conversion of food into body weight growth and is generally undesirable. A large or poor FCR is undesirable due to the cost of feed and the necessity to use more feed to grow fish to a given weight. The FCR values for fish subjected to the methods of the present invention are generally smaller and more desirable, in some instances (*e.g.*, when fish were fed dry feed), than most industry published values because the present invention eliminates the presence of osmotically damaged fish that tend to increase the overall FCR since they eat food but do not grow. The methods of the present invention, result in a lower FCR, allowing optimal feeding and growth of most fish. The FCR of fish subjected to the present invention is sufficient to maintain growth and feeding of the majority of fish, or preferably increase the growth and feed consumption of the majority of fish. When fish are subjected to the methods of the present invention, they exhibit ranges of FCRs, for example, would include values between about 0.7 and about 7.0. In particular, food consumption or food intake is improved because it is believed that the fish "smell" or "sense" the food with the PVCR in cells of the olfactory lamellae or olfactory bulb.

The specific growth rate (SGR) of the fish was determined by dividing the weight of the fish at the end of the given time point by the starting weight of the fish.

All calculations to obtain feed conversion ratio (FCR) or specific growth rate (SGR) and growth factor (GF3) were performed using standard accepted formulae

(Willoughby, S. Manual of Salmonid Farming Blackwell Scientific, Oxford UK 1999)

Results and Discussion:

A marine fish, Summer Flounder, can be adapted and grown under APS Process I conditions for a prolonged interval (51 days) with growth rates similar to that exhibited by matched control Summer Flounder in seawater.

Tables I and II display data obtained from identical groups of summer flounder maintained under either seawater (seawater control) or APS Process I freshwater conditions. Water quality and temperatures (16.3°C vs 17.9°C average) were comparable. Flounders were successfully adapted to APS Process I conditions without significant mortalities and their overall appearance did not differ significantly from those matched controls that were maintained in seawater.

Table I: Growth of Summer Flounder in Freshwater.

	Flounder #		APS Freshwater Weight Start	Weight 20 days	Weight 37 days	Weight 51 days	Total Weight Gained
	116	1	161	145	144	140	-21
	118	2	87	94			
5	123	3	60				
	142	4	94	104	115	112	18
	146	5	73	73			
	221	6	118	135	145	156	38
	223	7	105				
10	225	8	96	112	124	133	37
	226	9	156	183	203	221	65
	227	10	162	176	172	180	18
	233	11	205	207	220	244	39
	234	12	221	224			
15	235	13	150	161	164	174	24
	Average		129.8462	146.7273	160.875	170	27.2
	S.Dev.		50.15	48.34065	36.65452	44.75648	
	p test			0.017186	0.013243	0.0085	
	Amount Fed (gm)			342	315	291	948
20	water °C (Average)		17.9	19.4	16.4	17.9	

FCR 3.96

SGR 0.53% bw/day

Feed conversion ratio (FCR); specific growth rate (SGR)

Table II: Growth of Summer Flounder in Seawater.

Fish #	APS Seawater Weight Start	Total days 51 Weight 20 days	Weight 37 days	Weight 51 days	Total Weight Gained
117	114				
118	147	146	168	168	21
120	60	70	91	94	34
122	90	115	142	153	63
126	128	142	174	196	68
127	67	76	93	105	38
130	95	90	93	86	-9
131	92	87	101	104	12
132	93	101	121	127	34
134	174	191	235	236	62
139	116				
140	121	138	170	175	54
145	79	87	100	135	56
Average	105.8462	113	135.2727	143.5455	39.3636 4
S. Dev.	31.997	37.26392	46.82327	47.05181	
T test		0.308845	0.041076	0.014804	
Amount Fed (gm)		301	324	254	879

FCR 1.99

SGR 0.6%bw/ day

body weight=bw

Overall mortalities of fish during the 51 day test interval was lower in seawater (2/13 or 15.4%) as compared to flounders maintained under APS Process I conditions (5/13 or 38.5%). The average weight gained by all flounders maintain under APS Process I conditions (27.2 gm) was less as compared to overall weight gain of the seawater control group (38.4 gm). Significant weight gains were observed in both groups after intervals of 20 days for APS Process I fish and 37 days for flounder maintained in seawater. Thus, the average specific growth rates (SGR)

amongst the surviving flounders in APS Process I (0.53% body weight per day) were comparable to those maintained in seawater (0.6% body weight per day).

In contrast, 100% of marine fish (Cod, Haddock, Hake, Halibut, Mackerel, Pollock, Sea Bass, Swordfish, Tuna, Winter Flounder and Summer Flounder) die within 72 hours of freshwater transfer.

Comparison of the food conversion ratio (FCR) between flounders maintained in APS Process I *vs* seawater shows that flounders maintained under APS Process I conditions displayed a significantly greater FCR (3.96), as compared to their matched seawater controls (1.99).

Figures 1A-B show the individual weight gain performances of tagged flounders maintained under APS Process I or seawater conditions. It is notable that there are wide variations in individual growth rates such that some flounders (*e.g.* #9 and #11) exhibited steady and significant growth under APS Process I conditions while others showed poor weight gains (*e.g.* #10) or even lost weight (*e.g.* #1). Similar performance characteristics were observed for flounder in seawater although the variation in individual performances were less pronounced as compared to flounders maintained in APS Process I.

Taken together, these data demonstrate that summer flounder can be successfully maintained under freshwater conditions using APS Process I for a prolonged interval (51 days) of time. Under normal conditions, summer flounder growth and survival are normally restricted to approximately 25% seawater whereupon the flounders die if the salinity is further reduced. These data form the basis of culture of summer flounder in freshwater environments distant from the marine environment itself where prices for flounder fillets would more than offset the poorer performance (increased mortalities and poorer FCR and weight gains) as compared to seawater controls.

Transferring marine fish to freshwater using APS Process II is expected to provide even better growth rates, than seen with APS Process I. Salmon and Trout that underwent APS Process II exhibited significant increases in growth rates, as illustrated in related applications, Patent Application Nos. 09/687,372; 09/687,476; 09/687,477, all entitled, "Methods for Raising Pre-Adult Anadromous Fish," and

Patent Application No. 09/687,373, entitled "Growing Marine Fish in Fresh Water", all filed on October 12, 2000.

Example 3: The Feed

Two general methods were used to prepare feed for consumption by fish as part of APS Process I and II. These two processes involve either reformulation of feed or addition of a concentration solution for absorption by the feed followed by a top dressing for palatability. This disclosure describes the methodology to prepare feed using each of these 2 methods.

Methods:

Feed Manufacture for salmon experiments

To reformulate feed, the ingredients are as follows: Base Diet was made using the following ingredients and procedure: 30% Squid (liquefied in blender), 70% Corey Aquafeeds flounder diet (powderized in blender). Ingredients were blended into a semi moist "dough" ball. Other ingredients including NaCl or PVCR active compounds were blended into the base diet by weight according to experimental parameters.

Moore Clark standard freshwater salmonid diet (sizes 1.2,1.5, 2.0, 2.5, and 3.5 mm) can also be used. A top dressing was applied to the pellets such that top dressing is composed of 4% of the weight of the Base Diet. Top dressing is composed of 50% krill hydrolysate (Specialty Marine Products Ltd.) and 50% Menhaden fish oil. The top dressing is added for palatability and sealing of added ingredients

Other ingredients can include NaCl, $MgCl_2$, $CaCl_2$ or L-Tryptophan that are added by weight to the base diet by weight, as described herein.

Preparation of Feed Containing 7% (weight/weight) NaCl:

For the APS Process I: Solid NaCl or NaCl apportioned at a ratio of 7% of the weight of the Moore Clark standard freshwater salmonid diet weight was added to a volume of tap water approximately 3-4 times the weight of NaCl. The mixture

was heated to 60-70°C with mixing via use of a magnetic stirring bar to dissolve salt. The NaCl solution was then poured into a hand held sprayer and applied to the Moore Clark standard freshwater salmonid diet that is tumbling inside of a 1.5 cubic meter motorized cement mixer. After absorption of the NaCl rich solution, the wetted Moore Clark standard freshwater salmonid diet is spread out thinly on window screening and placed in an enclosed rack system equipped with a fan and 1500 watt heater to expedite drying process. After drying for approximately 6 hr, the dried NaCl-rich pellets are returned to the cement mixer and a top dressing is applied. The feed is stored at room temperature until use.

Preparation of Feed Containing 7% (weight/weight) NaCl + PVCR Agonist (Tryptophan) For the APS Process II: Solid sodium chloride or NaCl apportioned at a ratio of 7% of the weight of the Moore Clark standard freshwater salmonid diet weight was added to a volume of tap water approximately 3-4 times the weight of NaCl. The mixture was heated to 60-70°C with mixing via use of a magnetic stirring bar to dissolve salt. USP Grade L-Tryptophan was added to the water at either 2 grams or 4 grams for every kg of Moore Clark standard freshwater salmonid diet depending on formulation need. Dilute hydrochloric acid was added to the water with mixing until the tryptophan was dissolved and the pH of solution was approximately 4.0. The NaCl + tryptophan solution was then poured into a hand held sprayer and was then applied to the Moore Clark standard freshwater salmonid diet tumbling inside a cement mixer. After absorption of the NaCl + tryptophan solution, the wetted Moore Clark standard freshwater salmonid diet is then spread out thinly on window screening and placed in an enclosed rack system equipped with a fan and 1500-watt heater to expedite drying process. After drying for approximately 6 hr, the dried NaCl/tryptophan-rich pellets are then returned to the cement mixer and a top dressing is applied. The feed is stored at room temperature until use.

Example 4: DNA and Putative Protein Sequences from Partial Genomic Clones of Polyvalent Cation Receptor Protein Amplified by PCR from the DNA of Several Species of Marine fish.

These data provide the partial PVCR genomic sequences isolated in 13 species of marine fish. Each of these nucleotide sequences is unique and thus could be used as a unique probe to isolate the full-length cDNA from each species. Moreover, these nucleotide sequences could form the basis for a specific assay kit(s) for detection of PVCR expression in various tissues of these fish. For example, the kit could optionally include a labeled hybridization probe suitable for *in situ* hybridization.

The PVCR has been isolated in several species including Cod, Haddock, Hake, Halibut, Mackerel, Pollock, Sea Bass, Swordfish, Tuna, Winter Flounder and Summer Flounder. Sequences of mammalian CaRs together with the nucleotide sequence of SKCaR (Figures 14A and 14B) were used to design degenerate oligonucleotide primers to highly conserved regions in the extracellular and transmembrane domains of polyvalent cation receptor proteins using standard methodologies (See GM Preston, "Polymerase chain reaction with degenerate oligonucleotide primers to clone gene family members," *Methods in Mol. Biol. Vol. 58,* Edited by A. Harwood, Humana Press, pages 303-312, 1993). Using these primers, cDNA or genomic DNA from various fish species representing important commercial products are amplified using standard PCR methodology. Amplified bands are then purified by agarose gel electrophoresis and ligated into appropriate plasmid vector that is transformed into a bacterial strain. After growth in liquid media, vectors and inserts are purified using standard techniques, analyzed by restriction enzyme analysis and sequenced where appropriate. Using this methodology, nucleotide sequences were amplified.

To generate this sequence data, DNA was isolated from tissue samples of each of the species indicated using standard published techniques. DNA was then amplified using polymerase chain reaction (PCR) methodology including 2 degenerate PCR primers (DSK-F3 (5'-TGT CKT GGA CGG AGC CCT TYG GRA TCG C-3'; SEQ ID NO.: 29) and DSK-R4; (5'-GGC KGG RAT GAA RGA KAT CCA RAC RAT GAA G-3' SEQ ID NO:30). Amplified DNAs were then purified by agarose gel electrophoresis, subcloned into plasmid vectors, amplified, purified and sequenced using standard methods.

Figures 12A-C show an aligned genomic DNA sequences of 593 nucleotides for 12 marine fish species, each of which codes for an identical region of the PVCR protein. Note that each nucleotide sequence derived from each specific species is unique. However, alterations in the DNA sequences of these genes often occur at common specific nucleotides within each sequence of 593 nucleotides.

Figures 13A-C show aligned corresponding predicted protein sequences derived from genomic nucleotide sequences displayed in Figures 12A-D. Note that few alterations in the amino acid sequence of this portion of the PVCR occur as a consequence of alterations in the nucleotide sequence as shown in Figures 12A-D. All of these changes (*e.g.*, Ala to Val; Arg to Lys; and Cys to Tyr) are known as "conservative" substitutions of amino acids in that they preserve some combination of the relative size, charge and hydrophobicity of the peptide sequence.

All cited references, patents, and patent applications are incorporated herein by reference in their entirety. Also, companion Patent Application No. not yet assigned (Attorney Docket No. 2213.2004-001), entitled "Methods for Growing and Imprinting Fish Using Odorant," filed October 11, 2001; Patent Application No. not yet assigned (Attorney Docket No. 2213.1004-001), entitled "Methods for Raising Pre-adult Anadromous Fish," filed October 11, 2001; International Application No. not yet assigned (Attorney Docket No. 2213.1006-003), entitled "Polyvalent Cation-sensing Receptor Proteins in Aquatic Species," filed October 11, 2001. Additionally, Patent Application No. 09/687,477, entitled "Methods for Raising Marine Fish," filed on October 12, 2000; Patent Application No. 09/687,476, entitled "Methods for Raising Marine Fish," filed on October 12, 2000; Patent Application No. 09/687,373, entitled "Methods for Raising Marine Fish," filed on October 12, 2000; Provisional Patent Application No. 60/240,392, entitled "Polyvalent Cation Sensing Receptor Proteins in Aquatic Species," filed on October 12, 2000; Provisional Patent Application No. 60/240,003, entitled "Polyvalent Cation Sensing Receptor Proteins in Aquatic Species," filed on October 12, 2000, are all hereby incorporated by reference in their entirety. Additionally, Application No. 09/162,021, filed on September 28, 1998, International PCT application No. PCT/US97/05031, filed on March 27, 1997, and Application No. 08/622,738 filed

March 27, 1996, all entitled, " Polycation Sensing Receptor in Aquatic Species and Methods of Use Thereof" are all hereby incorporated by reference in their entirety.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes can be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method of growing marine fish in freshwater, comprising:
   a) adding at least one Polyvalent Cation Sensing Receptor (PVCR) modulator to freshwater in an amount sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR in one or more tissues;
   b) transferring the marine fish to the freshwater, modified according to step a); and
   c) adding feed for fish consumption to the modified freshwater, wherein the feed contains an amount of NaCl sufficient to contribute to a significant increased level of said PVCR modulator in serum of the marine fish.

2. The method of Claim 1, wherein the PVCR modulator is selected from the group consisting of a divalent cation, a trivalent cation, an aminoglycoside, an organic polycation, an amino acid, a Type I Calcimimetic, a Type II Calcimimetic, 1,25 dihydroxyvitamin D, a cytokine, and macrophage chemotatic peptide-1.

3. The method of Claim 2, wherein the feed contains at least about 1% NaCl by weight.

4. The method of Claim 1, wherein the feed contains a PVCR modulator.

5. A method of transferring marine fish to freshwater, comprising:
   a) adding at least one Polyvalent Cation Sensing Receptor (PVCR) modulator to the freshwater in an amount sufficient to modulate or

maintain expression and/or sensitivity of at least one PVCR in one or more tissues;

b) transferring the marine fish to the freshwater, modified according to step a); and

c) adding feed for fish consumption to the modified freshwater, wherein the feed contains at least about 1% NaCl by weight.

6. The method of Claim 5, wherein the PVCR modulator is a PVCR agonist.

7. The method of Claim 6, wherein the PVCR agonist is selected from the group consisting of a divalent cation, a trivalent cation, an aminoglycoside, an organic polycation and an amino acid.

8. A method of growing marine fish in freshwater, comprising:

a) determining the level of at least one PVCR modulator in freshwater;

b) based on the level determined in step a), adding said PVCR modulator to the freshwater in an amount sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR in one or more tissues;

c) transferring the marine fish to the freshwater, modified according to step b); and

d) adding feed for fish consumption to the modified freshwater, wherein the feed contains an amount of NaCl sufficient to contribute to a modulated level of said PVCR modulator in serum of the marine fish.

9. The method of Claim 8, wherein the PVCR modulator assessed is selected from the group consisting of calcium and magnesium.

10. The method of Claim 9, wherein the freshwater has between about 0.3 mM and 10.0 about mM calcium and between about 0.5 mM and about 10.0 mM magnesium prior to transferring marine fish.

11. A method of growing marine fish in freshwater having between about 0.3 mM and about 10.0 mM of calcium and between about 0.5 mM and 10.0 mM of magnesium, the method comprising adding feed to the freshwater wherein the feed contains an amount of NaCl sufficient to contribute to a significant increased level of said PVCR modulator in serum of the marine fish; wherein modulated expression of at least one PVCR occurs in one or more tissues.

12. The method of Claim 11, wherein the feed contains at least about 1% NaCl by weight.

13. A method of transferring marine fish to freshwater, comprising:

a) transferring the marine fish to freshwater having magnesium and calcium in the freshwater in amounts sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR in one or more tissues, and

b) adding feed to the freshwater, wherein the feed contains at least about 1% NaCl by weight.

14. A method of growing flounder in freshwater, comprising:

a) transferring the flounder to freshwater having at least one PVCR modulator in an amount sufficient to modulate or maintain expression and/or sensitivity of at least one PVCR in one or more tissue;

b) adding feed for fish consumption to the freshwater, wherein the feed contains an amount of NaCl sufficient to contribute to a significant increased level of said PVCR modulator in serum of the flounder.

15. The method of Claim 14, wherein the pH of the freshwater is greater than 7.0.

16. The method of Claim 14, wherein the PVCR modulator is selected from the group consisting of a divalent cation, a trivalent cation, an aminoglycoside, a organic polycation, an amino acid, a Type I Calcimimetic, a Type II Calcimimetic, 1,25 dihydroxyvitamin D, a cytokine, and macrophage chemotatic peptide-1.

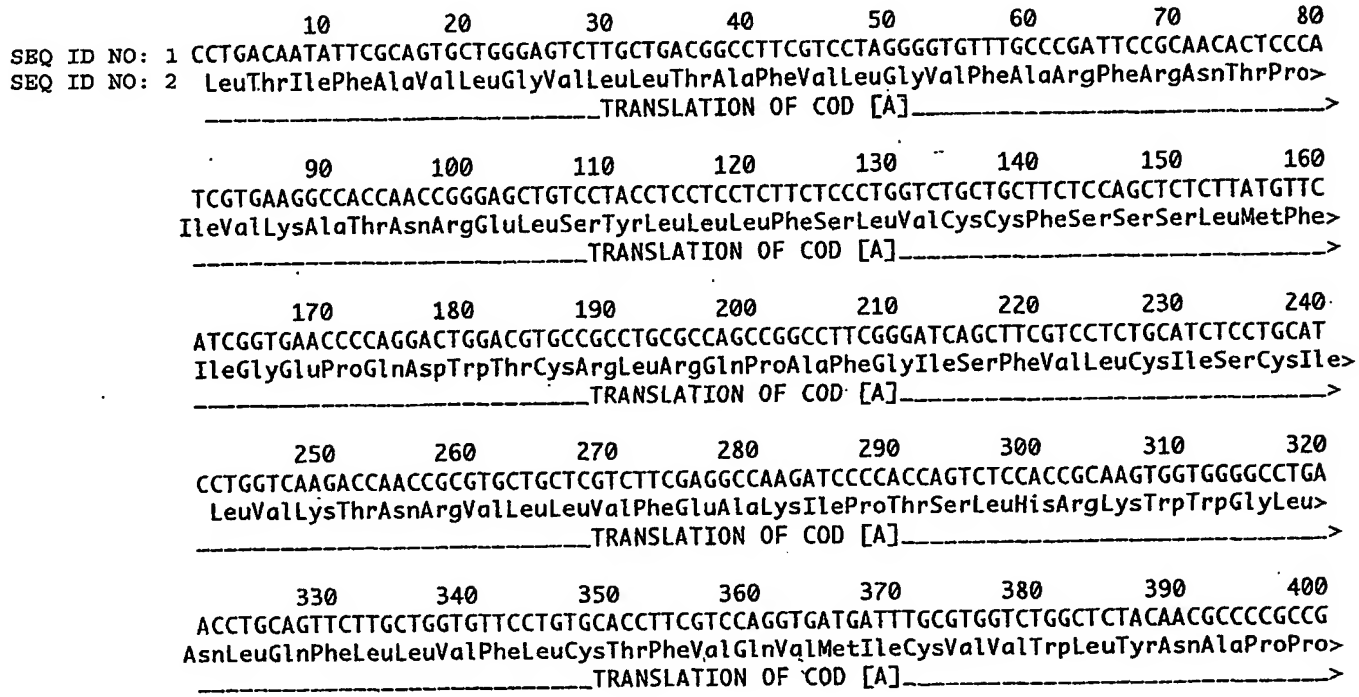
17. The method of Claim 16, wherein the feed comprises at least about 1% NaCl by weight.

18. An aquatic mixture for providing an environment to transfer marine fish to freshwater, comprising at least one PVCR modulator.

19. A kit for growing marine fish in freshwater, comprising:

a) an aquatic mixture for providing an environment to grow the marine fish, wherein the aquatic mixture comprises at least one PVCR modulator; and

b) an aquatic food composition containing a concentration of NaCl between about 10,000 mg/kg and about 100,000 mg/kg.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SEQ ID NO: 2 LeuThrIlePheAlaValLeuGlyValLeuLeuThrAlaPheValLeuGlyValPheAlaArgPheArgAsnThrPro>
TRANSLATION OF COD [A]
90 100 110 120 130 140 150 160
TCGTGAAGGCCACCAACCGGGAGCTGTCCTACCTCCTCCTCTTCTCCCTGGTCTGCTGCTTCTCCAGCTCTCTTATGTTC
IleValLysAlaThrAsnArgGluLeuSerTyrLeuLeuLeuPheSerLeuValCysCysPheSerSerSerLeuMetPhe>
TRANSLATION OF COD [A]
170 180 190 200 210 220 230 240
ATCGGTGAACCCCAGGACTGGACGTGCCGCCTGCGCCAGCCGGCCTTCGGGATCAGCTTCGTCCTCTGCATCTCCTGCAT
IleGlyGluProGlnAspTrpThrCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF COD [A]
250 260 270 280 290 300 310 320
CCTGGTCAAGACCAACCGCGTGCTGCTCGTCTTCGAGGCCAAGATCCCCACCAGTCTCCACCGCAAGTGGTGGGGCCTGA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF COD [A]
330 340 350 360 370 380 390 400
ACCTGCAGTTCTTGCTGGTGTTCCTGTGCACCTTCGTCCAGGTGATGATTTGCGTGGTCTGGCTCTACAACGCCCCGCCG
AsnLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
TRANSLATION OF COD [A]

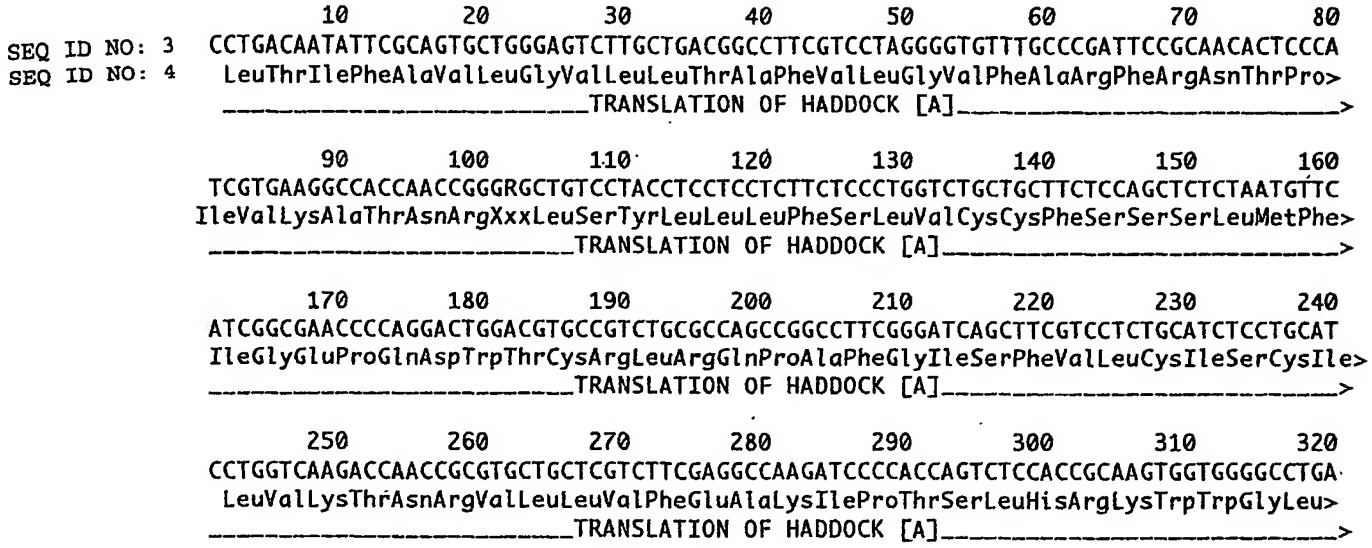
FIG. 1A

```
                    410       420       430       440       450       460       470       480
SEQ ID NO: 1  GCCAGCTCCAAGAACCACGACATCGATGAGATCATCTTCATCACCTGCAACGAGGGCTCCATGATGGCCCTGGGCTTTCT
SEQ ID NO: 2  AlaSerSerLysAsnHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerMetMetAlaLeuGlyPheLeu>
              ____________________________TRANSLATION OF COD [A]_____________________________>

                    490       500       510       520       530       540       550       560
              GATCGGCTACACCTGTCTCCTTGCCGCCATTTGCTTCTTCTTCGCGTTCAAATCGCGCAAACTCCCGGAGAACTTCACAG
               IleGlyTyrThrCysLeuLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
              ____________________________TRANSLATION OF COD [A]_____________________________>

                    570       580       590
              AGGCGAAGTTCATCACGTTTAGCATGCTGATATT
             GluAlaLysPheIleThrPheSerMetLeuIleXxx>
              _____TRANSLATION OF COD [A]_______>
```

FIG. 1B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SEQ ID NO: 3 CCTGACAATATTCGCAGTGCTGGGAGTCTTGCTGACGGCCTTCGTCCTAGGGGTGTTTGCCCGATTCCGCAACACTCCCA
SEQ ID NO: 4 LeuThrIlePheAlaValLeuGlyValLeuLeuThrAlaPheValLeuGlyValPheAlaArgPheArgAsnThrPro>
TRANSLATION OF HADDOCK [A]
90 100 110 120 130 140 150 160
TCGTGAAGGCCACCAACCGGGRGCTGTCCTACCTCCTCCTCTTCTCCCTGGTCTGCTGCTTCTCCAGCTCTCTAATGTTC
IleValLysAlaThrAsnArgXxxLeuSerTyrLeuLeuLeuPheSerLeuValCysCysPheSerSerSerLeuMetPhe>
TRANSLATION OF HADDOCK [A]
170 180 190 200 210 220 230 240
ATCGGCGAACCCCAGGACTGGACGTGCCGTCTGCGCCAGCCGGCCTTCGGGATCAGCTTCGTCCTCTGCATCTCCTGCAT
IleGlyGluProGlnAspTrpThrCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF HADDOCK [A]
250 260 270 280 290 300 310 320
CCTGGTCAAGACCAACCGCGTGCTGCTCGTCTTCGAGGCCAAGATCCCCACCAGTCTCCACCGCAAGTGGTGGGGCCTGA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF HADDOCK [A]

FIG. 2A

```
                330       340       350       360       370       380       390       400
SEQ ID NO: 3  ACCTGCAGTTCCTGCTGGTGTTCCTGTGCACCTTCGTCCAGGTGATGATTTGYGTGGTCTGGCTCTACAACGCCCCGCCG
SEQ ID NO: 4 AsnLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
              ____________________________TRANSLATION OF HADDOCK [A]_____________________________>

                410       420       430       440       450       460       470       480
              GCCAGCTCCAAGAACCACGACATTGATGAGATCATCTTCATCACCTGCAACGAGGGCTCCATGATGGCCCTGGGCTTTCT
              AlaSerSerLysAsnHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerMetMetAlaLeuGlyPheLeu>
              ____________________________TRANSLATION OF HADDOCK [A]_____________________________>

                490       500       510       520       530       540       550       560
              GATCGGCTACACCTGTCTCCTCGCCGCCATTTGCTTCTTCTTCGCGTTCAAATCGCGCAAACTCCCGGAGAACTTCACAG
               IleGlyTyrThrCysLeuLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
              ____________________________TRANSLATION OF HADDOCK [A]_____________________________>

                570       580       590
              AGGCGAAGTTCATCACGTTCAGCATGCTGATATT
             GluAlaLysPheIleThrPheSerMetLeuIleXxx>
              ___TRANSLATION OF HADDOCK [A]_____>
```

FIG. 2B

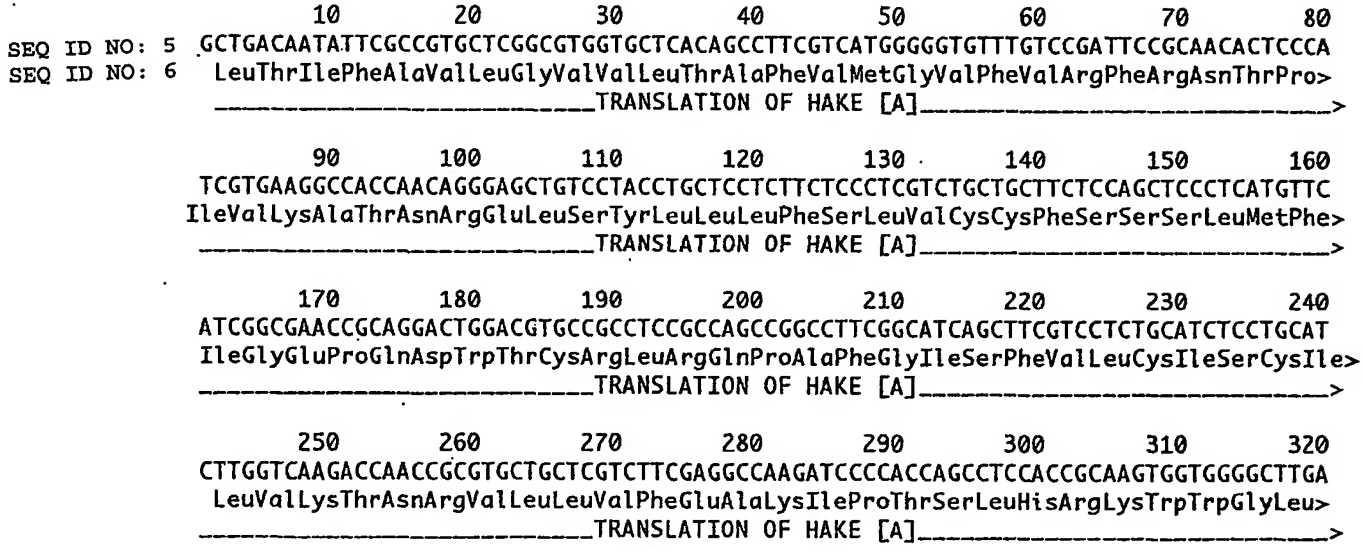

10 20 30 40 50 60 70 80
SEQ ID NO: 5 GCTGACAATATTCGCCGTGCTCGGCGTGGTGCTCACAGCCTTCGTCATGGGGGTGTTTGTCCGATTCCGCAACACTCCCA
SEQ ID NO: 6 LeuThrIlePheAlaValLeuGlyValValLeuThrAlaPheValMetGlyValPheValArgPheArgAsnThrPro>
TRANSLATION OF HAKE [A]
90 100 110 120 130 140 150 160
TCGTGAAGGCCACCAACAGGGAGCTGTCCTACCTGCTCCTCTTCTCCCTCGTCTGCTGCTTCTCCAGCTCCCTCATGTTC
IleValLysAlaThrAsnArgGluLeuSerTyrLeuLeuLeuPheSerLeuValCysCysPheSerSerSerLeuMetPhe>
TRANSLATION OF HAKE [A]
170 180 190 200 210 220 230 240
ATCGGCGAACCGCAGGACTGGACGTGCCGCCTCCGCCAGCCGGCCTTCGGCATCAGCTTCGTCCTCTGCATCTCCTGCAT
IleGlyGluProGlnAspTrpThrCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF HAKE [A]
250 260 270 280 290 300 310 320
CTTGGTCAAGACCAACCGCGTGCTGCTCGTCTTCGAGGCCAAGATCCCCACCAGCCTCCACCGCAAGTGGTGGGGCTTGA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF HAKE [A]

FIG. 3A

```
                330       340       350       360       370       380       390       400
SEQ ID NO: 5    ACCTGCAGTTCCTGCTGGTGTTCCTGTGCACCTTTGTCCAGGTGATGATCTGCGTGGTGTGGCTGTACAACGCCCCGCCG
SEQ ID NO: 6   AsnLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
                ______________________________TRANSLATION OF HAKE [A]______________________________>

                410       420       430       440       450       460       470       480
                GCCAGCTCCAAGAACCACGACATCGATGAGATCATCTTCATCACCTGCAACGAGGGCTCCATGATGGCCCTGGGCTTTCT
                AlaSerSerLysAsnHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerMetMetAlaLeuGlyPheLeu>
                ______________________________TRANSLATION OF HAKE [A]______________________________>

                490       500       510       520       530       540       550       560
                GATCGGCTACACTTGCATCCTCGCCGCTATCTGCTTCTTCTTCGCGTTCAAGTCGCGCAAACTCCCCGAGAACTTCACGG
                 IleGlyTyrThrCysIleLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
                ______________________________TRANSLATION OF HAKE [A]______________________________>

                570       580       590
                AGGCCAAGTTCATCACGTTCAGCATGCTGATATT
               GluAlaLysPheIleThrPheSerMetLeuIleXxx>
                _____TRANSLATION OF HAKE [A]______>
```

FIG. 3B

```
                10        20        30        40        50        60        70        80
SEQ ID NO: 7    GTTGACCATATGTGCGGCGCTGGGTGTTGCCTTGACAGGCTTCGTGATGGCCGTCTTTGTCAGATTCCGCAACACCCCCA
SEQ ID NO: 8     LeuThrIleCysAlaAlaLeuGlyValAlaLeuThrGlyPheValMetAlaValPheValArgPheArgAsnThrPro>
                 ------------------------TRANSLATION OF HALIBUT [A]------------------------->

               90        100       110       120       130       140       150       160
                TAGTGAAGGCCACGAACCGAGAACTGTCCTACGTCCTCCTGTTCTCTCTCATCTGTTGCTTCTCCAGCTCCCTCATCTTC
               IleValLysAlaThrAsnArgGluLeuSerTyrValLeuLeuPheSerLeuIleCysCysPheSerSerSerLeuIlePhe>
                ------------------------TRANSLATION OF HALIBUT [A]------------------------->

              170       180       190       200       210       220       230       240
               ATAGGAGAGCCGCAGGATTGGATGTGCCGCTTACGCCAACCTGCCTTTGGGATCAGTTTTGTTCTCTGTATCTCGTGCAT
               IleGlyGluProGlnAspTrpMetCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
               ------------------------TRANSLATION OF HALIBUT [A]------------------------->

              250       260       270       280       290       300       310       320
               CCTTGTCAAAACAAACAGAGTCCTCTTGGTGTTTGAAGCCAAGATCCCTACAAGTCTCCATCGTAAAYGGTGGGGGTTAA
                LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysXxxTrpGlyLeu>
               ------------------------TRANSLATION OF HALIBUT [A]------------------------->
```

FIG. 4A

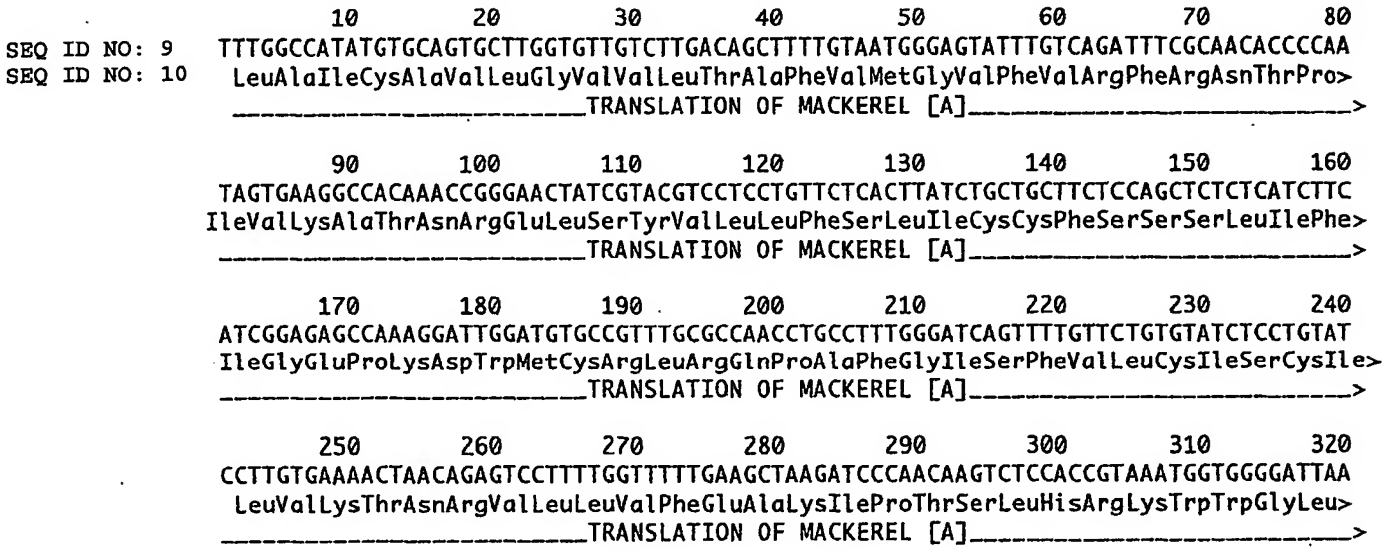
```
                330       340       350       360       370       380       390       400
SEQ ID NO: 7    RCCTACAGTTCCTGCTGGTGTTTCTGTGCACATTTGTCCAAGTCATGATATGTGTGGTCTGGCTGTACAACGCCCCACCT
SEQ ID NO: 8    XxxLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
                +-------------------------TRANSLATION OF HALIBUT [A]-------------------------->

                410       420       430       440       450       460       470       480
                TCCAGTTACAGGAATTATGACATAGATGAGATGATTTTTATCACATGTAACGAGGGCTCTGTAATGGCTCTTGGGTTTCT
                SerSerTyrArgAsnTyrAspIleAspGluMetIlePheIleThrCysAsnGluGlySerValMetAlaLeuGlyPheLeu>
                -------------------------TRANSLATION OF HALIBUT [A]-------------------------->

                490       500       510       520       530       540       550       560
                TATTGGCTATACATGCCTGCTGGCCGCTATAYGTTTCTTCTTTGCGTTTAAATCACGGAAACTTCCAGAAAACTTCACAG
                 IleGlyTyrThrCysLeuLeuAlaAlaIleXxxPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
                -------------------------TRANSLATION OF HALIBUT [A]-------------------------->

                570       580       590
                AGGCTAAGTTCATCACTTTTAGTATGCTCATATT
                GluAlaLysPheIleThrPheSerMetLeuIleXxx>
                ---TRANSLATION OF HALIBUT [A]----->
```

FIG. 4B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SEQ ID NO: 10 LeuAlaIleCysAlaValLeuGlyValValLeuThrAlaPheValMetGlyValPheValArgPheArgAsnThrPro>
TRANSLATION OF MACKEREL [A]
90 100 110 120 130 140 150 160
TAGTGAAGGCCACAAACCGGGAACTATCGTACGTCCTCCTGTTCTCACTTATCTGCTGCTTCTCCAGCTCTCTCATCTTC
IleValLysAlaThrAsnArgGluLeuSerTyrValLeuLeuPheSerLeuIleCysCysPheSerSerSerLeuIlePhe>
TRANSLATION OF MACKEREL [A]
170 180 190 200 210 220 230 240
ATCGGAGAGCCAAAGGATTGGATGTGCCGTTTGCGCCAACCTGCCTTTGGGATCAGTTTTGTTCTGTGTATCTCCTGTAT
IleGlyGluProLysAspTrpMetCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF MACKEREL [A]
250 260 270 280 290 300 310 320
CCTTGTGAAAACTAACAGAGTCCTTTTGGTTTTTGAAGCTAAGATCCCAACAAGTCTCCACCGTAAATGGTGGGGATTAA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF MACKEREL [A]

FIG. 5A

```
                330       340       350       360       370       380       390       400
SEQ ID NO: 9    ACCTGCAGTTTCTTTTGGTGTTTCTCTGCACATTTGTCCAAGTAATGATATGTGTGGTTTGGCTTTACAACGCCCCTCCT
SEQ ID NO: 10   AsnLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
                ___________________TRANSLATION OF MACKEREL [A]_____________________________>

                  410       420       430       440       450       460       470       480
                TCCAGTTATATGATCCATGACATTGATGAGATAATTTTTATCACCTGCAATGAGGGCTCTGTGATGGCTCTTGGCTTTCT
                SerSerTyrMetIleHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerValMetAlaLeuGlyPheLeu>
                ___________________TRANSLATION OF MACKEREL [A]_____________________________>

                  490       500       510       520       530       540       550       560
                TATTGGCTACACCTGCCTCCTGGCAGCTATATGTTTCTTTGCATTTAAATCACGAAAACTTCCAGAAAACTTTACAG
                  IleGlyTyrThrCysLeuLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
                ___________________TRANSLATION OF MACKEREL [A]_____________________________>

                  570       580       590
                AAGCCAAGTTCATCACTTTTAGCATGCTCATATT
                GluAlaLysPheIleThrPheSerMetLeuIleXxx>
                ___TRANSLATION OF MACKEREL [A]___>
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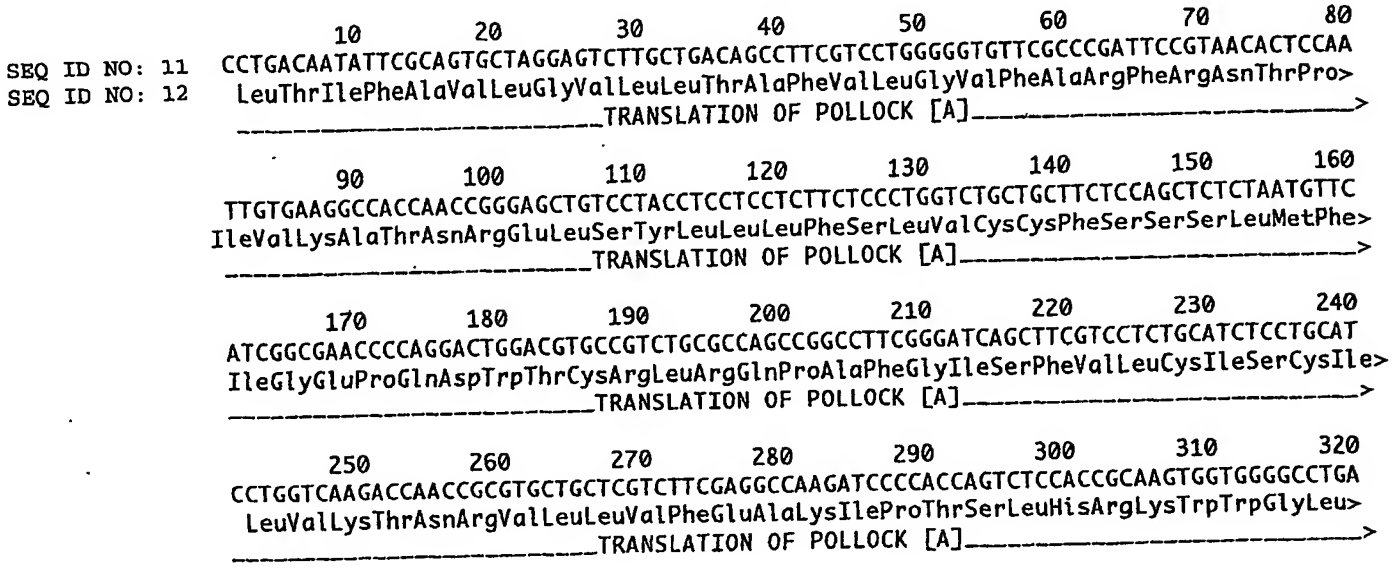
FIG. 5B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SEQ ID NO: 12 LeuThrIlePheAlaValLeuGlyValLeuLeuThrAlaPheValLeuGlyValPheAlaArgPheArgAsnThrPro>
TRANSLATION OF POLLOCK [A]
90 100 110 120 130 140 150 160
TTGTGAAGGCCACCAACCGGGAGCTGTCCTACCTCCTCCTCTTCTCCCTGGTCTGCTGCTTCTCCAGCTCTCTAATGTTC
IleValLysAlaThrAsnArgGluLeuSerTyrLeuLeuLeuPheSerLeuValCysCysPheSerSerSerLeuMetPhe>
TRANSLATION OF POLLOCK [A]
170 180 190 200 210 220 230 240
ATCGGCGAACCCCAGGACTGGACGTGCCGTCTGCGCCAGCCGGCCTTCGGGATCAGCTTCGTCCTCTGCATCTCCTGCAT
IleGlyGluProGlnAspTrpThrCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF POLLOCK [A]
250 260 270 280 290 300 310 320
CCTGGTCAAGACCAACCGCGTGCTGCTCGTCTTCGAGGCCAAGATCCCCACCAGTCTCCACCGCAAGTGGTGGGGCCTGA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF POLLOCK [A]

FIG. 6A

```
                330       340       350       360       370       380       390       400
SEQ ID NO: 11   ACCTGCAGTTCCTGCTGGTGTTCCTGTGCACCTTCGTCCAGGTGATGATTTGCGTGGTCTGGCTCTACAACGCCCCGCCG
SEQ ID NO: 12   AsnLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
                ____________________TRANSLATION OF POLLOCK [A]____________________>

                410       420       430       440       450       460       470       480
                GCCAGCTCCAAGAACCACGACATCGATGAGATCATCTTCATCACCTGCAACGAGGGCTCCATGATGGCCCTGGGCTTTCT
                AlaSerSerLysAsnHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerMetMetAlaLeuGlyPheLeu>
                ____________________TRANSLATION OF POLLOCK [A]____________________>

                490       500       510       520       530       540       550       560
                GATCGGCTACACCTGTCTCCTCGCCGCCATTTGCTTCTTCTTCGCGTTCAAATCGCGCAAACTCCCGGAGAACTTCACAG
                 IleGlyTyrThrCysLeuLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
                ____________________TRANSLATION OF POLLOCK [A]____________________>

                570       580       590
                AGGCGAAGTTCATCACGTTCAGCATGCTGATATT
                GluAlaLysPheIleThrPheSerMetLeuIleXxx>
                ___TRANSLATION OF POLLOCK [A]_____>
```

FIG. 6B

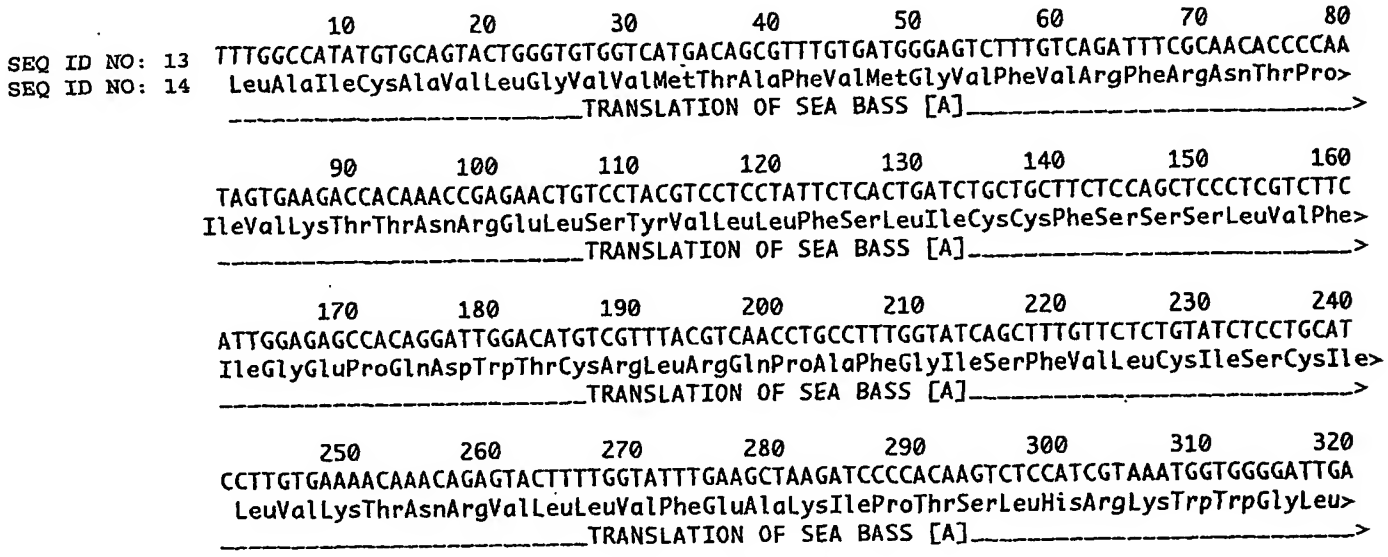

10 20 30 40 50 60 70 80
SEQ ID NO: 13 TTTGGCCATATGTGCAGTACTGGGTGTGGTCATGACAGCGTTTGTGATGGGAGTCTTTGTCAGATTTCGCAACACCCCAA
SEQ ID NO: 14 LeuAlaIleCysAlaValLeuGlyValValMetThrAlaPheValMetGlyValPheValArgPheArgAsnThrPro>
TRANSLATION OF SEA BASS [A]
90 100 110 120 130 140 150 160
TAGTGAAGACCACAAACCGAGAACTGTCCTACGTCCTCCTATTCTCACTGATCTGCTGCTTCTCCAGCTCCCTCGTCTTC
IleValLysThrThrAsnArgGluLeuSerTyrValLeuLeuPheSerLeuIleCysCysPheSerSerSerLeuValPhe>
TRANSLATION OF SEA BASS [A]
170 180 190 200 210 220 230 240
ATTGGAGAGCCACAGGATTGGACATGTCGTTTACGTCAACCTGCCTTTGGTATCAGCTTTGTTCTCTGTATCTCCTGCAT
IleGlyGluProGlnAspTrpThrCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF SEA BASS [A]
250 260 270 280 290 300 310 320
CCTTGTGAAAACAAACAGAGTACTTTTGGTATTTGAAGCTAAGATCCCCACAAGTCTCCATCGTAAATGGTGGGGATTGA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF SEA BASS [A]

FIG. 7A

```
                    330       340       350       360       370       380       390       400
SEQ ID NO: 13   ACCTGCAGTTCCTGCTGGTGTTTCTGTGCACATTTGTCCAAGTCATGATATGTGTGGTATGGCTTTACAACGCCCCTCCT
SEQ ID NO: 14 AsnLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
                ____________________TRANSLATION OF SEA BASS [A]____________________>

                    410       420       430       440       450       460       470       480
                TCCAGCTACAGGAATCACGACATTGATGAAATCATTTTTATCACCTGCAATGAGGGATCTGTGATGGCTCTTGGGTTTCT
                SerSerTyrArgAsnHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerValMetAlaLeuGlyPheLeu>
                ____________________TRANSLATION OF SEA BASS [A]____________________>

                    490       500       510       520       530       540       550       560
                TATTGGCCACACGTGCCTCCTGGCAGCTATATGTTTTTTCTTTGCATTCAAATCTCGGAAACTTCCAGAAAACTTTACAG
                 IleGlyHisThrCysLeuLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
                ____________________TRANSLATION OF SEA BASS [A]____________________>

                    570       580       590
                AGGCAAAGTTCATCACCTTTAGCATGCTAATATT
               GluAlaLysPheIleThrPheSerMetLeuIleXxx>
                ___TRANSLATION OF SEA BASS [A]____>
```

FIG. 7B

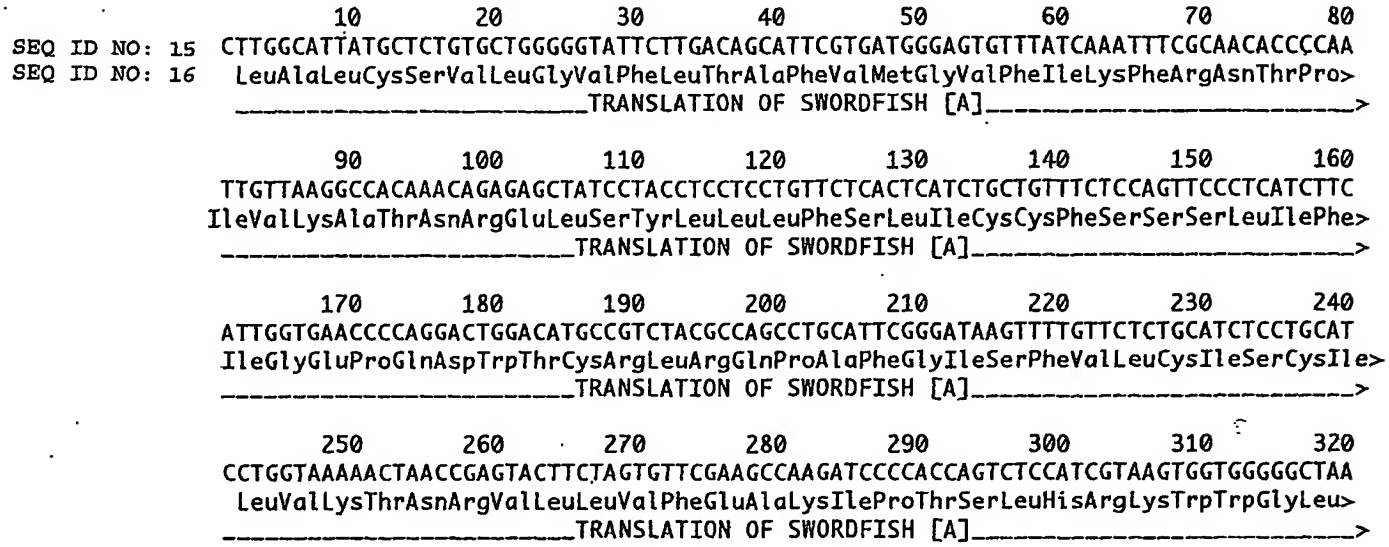

10 20 30 40 50 60 70 80
SEQ ID NO: 15 CTTGGCATTATGCTCTGTGCTGGGGGTATTCTTGACAGCATTCGTGATGGGAGTGTTTATCAAATTTCGCAACACCCCAA
SEQ ID NO: 16 LeuAlaLeuCysSerValLeuGlyValPheLeuThrAlaPheValMetGlyValPheIleLysPheArgAsnThrPro>
TRANSLATION OF SWORDFISH [A]
90 100 110 120 130 140 150 160
TTGTTAAGGCCACAAACAGAGAGCTATCCTACCTCCTCCTGTTCTCACTCATCTGCTGTTTCTCCAGTTCCCTCATCTTC
IleValLysAlaThrAsnArgGluLeuSerTyrLeuLeuLeuPheSerLeuIleCysCysPheSerSerSerLeuIlePhe>
TRANSLATION OF SWORDFISH [A]
170 180 190 200 210 220 230 240
ATTGGTGAACCCCAGGACTGGACATGCCGTCTACGCCAGCCTGCATTCGGGATAAGTTTTGTTCTCTGCATCTCCTGCAT
IleGlyGluProGlnAspTrpThrCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF SWORDFISH [A]
250 260 270 280 290 300 310 320
CCTGGTAAAAACTAACCGAGTACTTCTAGTGTTCGAAGCCAAGATCCCCACCAGTCTCCATCGTAAGTGGTGGGGGCTAA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF SWORDFISH [A]

FIG. 8A

```
                330       340       350       360       370       380       390       400
SEQ ID NO: 15   ACTTGCAGTTCCTGTTAGTGTTCCTGTTCACATTTGTGCAAGTGATGATATGTGTGGTCTGGCTTTACAATGCTCCTCCG
SEQ ID NO: 16  AsnLeuGlnPheLeuLeuValPheLeuPheThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
                ------------------------TRANSLATION OF SWORDFISH [A]-------------------------->

                410       420       430       440       450       460       470       480
                GCGAGCTACAGGAACCATGACATTGATGAGATAATTTTCATTACATGCAATGAGGGCTCTATGATGGCGCTTGGCTTCCT
                AlaSerTyrArgAsnHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerMetMetAlaLeuGlyPheLeu>
                ------------------------TRANSLATION OF SWORDFISH [A]-------------------------->

                490       500       510       520       530       540       550       560
                AATTGGGTACACATGCCTGCTGGCAGCCATATGCTTCTTCTTTGCATTTAAATCACGAAAACTGCCAGAGAACTTTACTG
                 IleGlyTyrThrCysLeuLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
                ------------------------TRANSLATION OF SWORDFISH [A]-------------------------->

                570       580       590
                AGGCTAAGTTCATCACCTTCAGCATGCTCATCTT
               GluAlaLysPheIleThrPheSerMetLeuIleXxx>
                ___TRANSLATION OF SWORDFISH [A____>
```

FIG. 8B

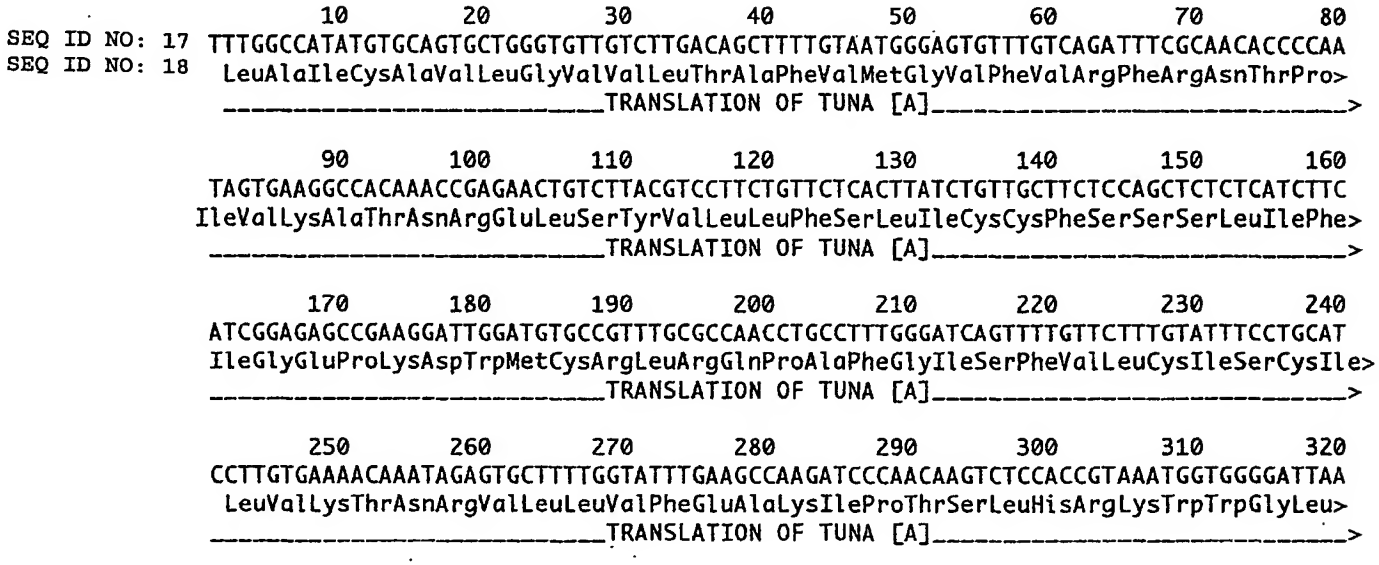

10 20 30 40 50 60 70 80
SEQ ID NO: 17 TTTGGCCATATGTGCAGTGCTGGGTGTTGTCTTGACAGCTTTTGTAATGGGAGTGTTTGTCAGATTTCGCAACACCCCAA
SEQ ID NO: 18 LeuAlaIleCysAlaValLeuGlyValValLeuThrAlaPheValMetGlyValPheValArgPheArgAsnThrPro>
TRANSLATION OF TUNA [A]
90 100 110 120 130 140 150 160
TAGTGAAGGCCACAAACCGAGAACTGTCTTACGTCCTTCTGTTCTCACTTATCTGTTGCTTCTCCAGCTCTCTCATCTTC
IleValLysAlaThrAsnArgGluLeuSerTyrValLeuLeuPheSerLeuIleCysCysPheSerSerSerLeuIlePhe>
TRANSLATION OF TUNA [A]
170 180 190 200 210 220 230 240
ATCGGAGAGCCGAAGGATTGGATGTGCCGTTTGCGCCAACCTGCCTTTGGGATCAGTTTTGTTCTTTGTATTTCCTGCAT
IleGlyGluProLysAspTrpMetCysArgLeuArgGlnProAlaPheGlyIleSerPheValLeuCysIleSerCysIle>
TRANSLATION OF TUNA [A]
250 260 270 280 290 300 310 320
CCTTGTGAAAACAAATAGAGTGCTTTTGGTATTTGAAGCCAAGATCCCAACAAGTCTCCACCGTAAATGGTGGGGATTAA
LeuValLysThrAsnArgValLeuLeuValPheGluAlaLysIleProThrSerLeuHisArgLysTrpTrpGlyLeu>
TRANSLATION OF TUNA [A]

FIG. 9A

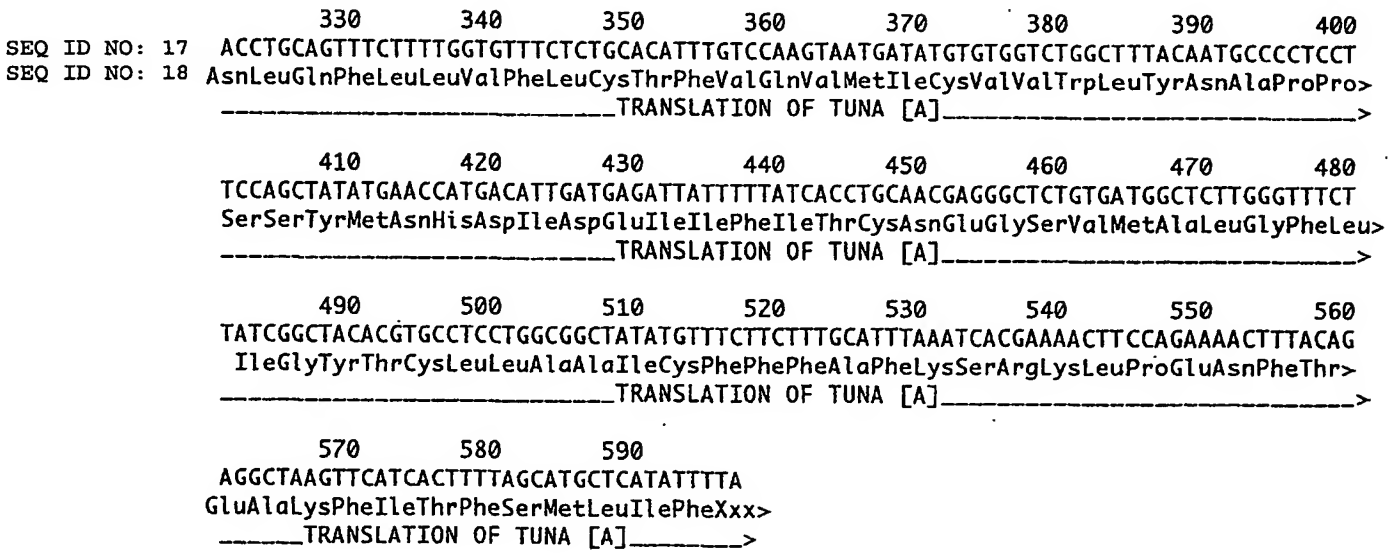

330 340 350 360 370 380 390 400
SEQ ID NO: 17 ACCTGCAGTTTCTTTTGGTGTTTCTCTGCACATTTGTCCAAGTAATGATATGTGTGGTCTGGCTTTACAATGCCCCTCCT
SEQ ID NO: 18 AsnLeuGlnPheLeuLeuValPheLeuCysThrPheValGlnValMetIleCysValValTrpLeuTyrAsnAlaProPro>
TRANSLATION OF TUNA [A]
410 420 430 440 450 460 470 480
TCCAGCTATATGAACCATGACATTGATGAGATTATTTTTATCACCTGCAACGAGGGCTCTGTGATGGCTCTTGGGTTTCT
SerSerTyrMetAsnHisAspIleAspGluIleIlePheIleThrCysAsnGluGlySerValMetAlaLeuGlyPheLeu>
TRANSLATION OF TUNA [A]
490 500 510 520 530 540 550 560
TATCGGCTACACGTGCCTCCTGGCGGCTATATGTTTCTTCTTTGCATTTAAATCACGAAAACTTCCAGAAAACTTTACAG
IleGlyTyrThrCysLeuLeuAlaAlaIleCysPhePhePheAlaPheLysSerArgLysLeuProGluAsnPheThr>
TRANSLATION OF TUNA [A]
570 580 590
AGGCTAAGTTCATCACTTTTAGCATGCTCATATTTTA
GluAlaLysPheIleThrPheSerMetLeuIlePheXxx>
TRANSLATION OF TUNA [A]

FIG. 9B

```
ccacgcgtcc gggagatggt caggagaaac atcacagatc gcatttggct agccagcgaa 60  SEQ ID NO. 19
gcgtgggcca gctcttccct tattgccaaa ccagagtatc ttgacgttgt ggcaggaact 120
attggctttg ctctgaaggc agggggtata ccaggcttta gggagttctt acaacatgtc 180
caaccaaaga aagacagtca taatgaattt gtcagggagt tttgggaaga aaccttcaac 240
tgttatctgg aggacagccc aagactgcaa gaatgtggca gcactagttt caggcctttg 300
tgcacaggtg aggaagacat cacaagcgtc gagaccccgt acctggactt cacacacctt 360
cgaatctcct ataatgtata tgttgcagtg tattccattg cacaggccct gcaggacatt 420
cttacctgca cacctggaca tggacttttt gccaacaatt cctgtgcaga tataaagaaa 480
atggaagcct ggcaggtcct gaagcagctg agacatttaa actacaccaa cagtatgggg 540
gaaaagatcc actttgatga gaatgacgac ctggctgcaa actacatgat cataaactgg 600
cacaggtcca ctgaagacgg ctctgtggtg ttcgaggagg ttggatacta ccacatgcac 660
gcgaagagag gggccaaact gctcattgac aggacaaaga ttctgtggaa tggatacagt 720
tcagaggtgc cattctcgaa ctgcagtgag gactgtgatc ctggcacaag aaagggcatc 780
atagatagta tgcccacatg ctgctttgaa tgcactgagt gctcagatgg agaatacagt 840
actcacaaag atgccagtgt ttgcaccaag tgtccaaata actcctggtc caatgggaac 900
cacacgttct gcttcctgaa ggaaattgag tttctctcct ggacagaacc tttcgggata 960
gcgttgacca tatgtgcagt gctgggtgtt gccctgacgg gcttcgtgat ggccgtcttt 1020
gtccgattcc gcaacacccc aatagtgaaa gccacgaacc gagaactgtc ctacgtcctc 1080
ctgttctctc tcatctgttg cttctccagc tccctcatct tcataggaga gccgcaggat 1140
tggatgtgcc gcttacgcca accggccttt gggatcagtt ttgttctctg tatctcgtgc 1200
atccttgtga aaacaaaccg agtcctcttg gtgtttgaag ccaagatccc gacaagtctc 1260
catcgtaaat ggtgggggtt aaacctacag ttcctgctgg tgtttctgtg cacatttgtc 1320
caagtcatga tatgtgtggt ctggctgtac aacgccccac cttccagtta caggaattat 1380
gacatagatg agatgatttt tatcacatgt aatgaaggct ctgtaatggc tcttgggttt 1440
cttattggct atacatgcct gctggccgct atatgtttct tctttgcatt caaatcacgg 1500
aaacttccag aaaacttcac cgaggctaag ttcatcactt ttagtatgct catattcttt 1560
atcgtttgga tctctttcat ccctgcctac ttcagtactt acggaaagtt tgtttcagcg 1620
gtggaggtca ttgccatact ggcctccagc tttgggatgc tggcctgcat cttcttcaac 1680
aaggtctaca tcatcctttt caaaccgtcc cggaacacca tcgaggaggt ccggtgcagc 1740
acctcagccc acgctttcaa agtggcggca aaggctactc taaagcatag cacggcttca 1800
cggagaaagt cgggcagcac tggtggatct tctgactcca cgccgtcatc gtccatcagc 1860
ctgaagacca atggcaatga cccgacttca ggaaagccca gggtgagctt tggcagtgga 1920
acagttactt tgtccttgag cttcgaggag tcgaggagga gttctctgat gtgatagaat 1980
atgtgtggct ctgtcaagtt tcagcttcat ctgtgtcatt aatagggtgt tttgttttgt 2040
tttttacacg taaaccttta catctttcct ttttcctaac attttgtccg gaatatgatc 2100
atcactccaa ctaatatact gcacctgaat cctgtgtctt gttaatgtgt agtaaatctg 2160
ctagtaatat tcacaaaacg ttttgtacaa ttaaaaaact ttatatgatc aaaaaaaaaa 2220
aaaaaaaag                                                        2229
```

FIG. 10A

SEQ ID NO. 20

```
Pro Arg Val Arg Glu Met Val Arg Arg Asn Ile Thr Asp Arg Ile Trp
 1               5                  10                  15
Leu Ala Ser Glu Ala Trp Ala Ser Ser Ser Leu Ile Ala Lys Pro Glu
            20                  25                  30
Tyr Leu Asp Val Val Ala Gly Thr Ile Gly Phe Ala Leu Lys Ala Gly
        35                  40                  45
Gly Ile Pro Gly Phe Arg Glu Phe Leu Gln His Val Gln Pro Lys Lys
    50                  55                  60
Asp Ser His Asn Glu Phe Val Arg Glu Phe Trp Glu Glu Thr Phe Asn
65                  70                  75                  80
Cys Tyr Leu Glu Asp Ser Pro Arg Leu Gln Glu Cys Gly Ser Thr Ser
                85                  90                  95
Phe Arg Pro Leu Cys Thr Gly Glu Glu Asp Ile Thr Ser Val Glu Thr
            100                 105                 110
Pro Tyr Leu Asp Phe Thr His Leu Arg Ile Ser Tyr Asn Val Tyr Val
        115                 120                 125
Ala Val Tyr Ser Ile Ala Gln Ala Leu Gln Asp Ile Leu Thr Cys Thr
    130                 135                 140
Pro Gly His Gly Leu Phe Ala Asn Asn Ser Cys Ala Asp Ile Lys Lys
145                 150                 155                 160
Met Glu Ala Trp Gln Val Leu Lys Gln Leu Arg His Leu Asn Tyr Thr
                165                 170                 175
Asn Ser Met Gly Glu Lys Ile His Phe Asp Glu Asn Asp Asp Leu Ala
            180                 185                 190
Ala Asn Tyr Met Ile Ile Asn Trp His Arg Ser Thr Glu Asp Gly Ser
        195                 200                 205
Val Val Phe Glu Glu Val Gly Tyr Tyr His Met His Ala Lys Arg Gly
    210                 215                 220
Ala Lys Leu Leu Ile Asp Arg Thr Lys Ile Leu Trp Asn Gly Tyr Ser
225                 230                 235                 240
Ser Glu Val Pro Phe Ser Asn Cys Ser Glu Asp Cys Asp Pro Gly Thr
                245                 250                 255
Arg Lys Gly Ile Ile Asp Ser Met Pro Thr Cys Cys Phe Glu Cys Thr
            260                 265                 270
Glu Cys Ser Asp Gly Glu Tyr Ser Thr His Lys Asp Ala Ser Val Cys
        275                 280                 285
Thr Lys Cys Pro Asn Asn Ser Trp Ser Asn Gly Asn His Thr Phe Cys
    290                 295                 300
Phe Leu Lys Glu Ile Glu Phe Leu Ser Trp Thr Glu Pro Phe Gly Ile
305                 310                 315                 320

Ala Leu Thr Ile Cys Ala Val Leu Gly Val Ala Leu Thr Gly Phe Val
            325                 330                 335
Met Ala Val Phe Val Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr
        340                 345                 350
Asn Arg Glu Leu Ser Tyr Val Leu Leu Phe Ser Leu Ile Cys Cys Phe
    355                 360                 365
Ser Ser Ser Leu Ile Phe Ile Gly Glu Pro Gln Asp Trp Met Cys Arg
370                 375                 380
Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys
385                 390                 395                 400
Ile Leu Val Lys Thr Asn Arg Val Leu Leu Val Phe Glu Ala Lys Ile
                405                 410                 415
Pro Thr Ser Leu His Arg Lys Trp Trp Gly Leu Asn Leu Gln Phe Leu
            420                 425                 430
Leu Val Phe Leu Cys Thr Phe Val Gln Val Met Ile Cys Val Val Trp
        435                 440                 445
```

FIG. 10B

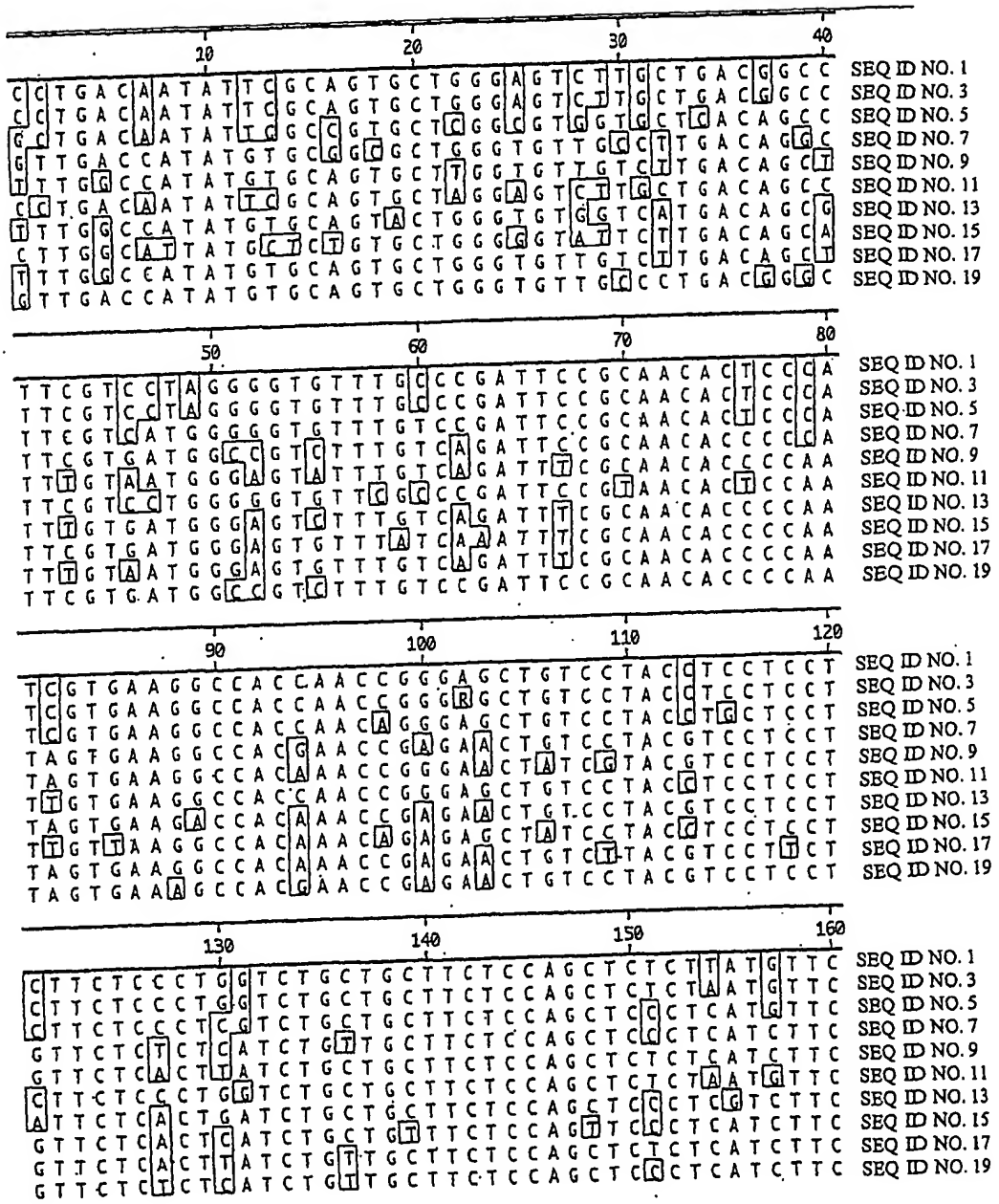
```
Leu Tyr Asn Ala Pro Pro Ser Ser Tyr Arg Asn Tyr Asp Ile Asp Glu SEQ ID NO. 20
    450                 455                 460
Met Ile Phe Ile Thr Cys Asn Glu Gly Ser Val Met Ala Leu Gly Phe
465                 470                 475                 480
Leu Ile Gly Tyr Thr Cys Leu Leu Ala Ala Ile Cys Phe Phe Phe Ala
                485                 490                 495
Phe Lys Ser Arg Lys Leu Pro Glu Asn Phe Thr Glu Ala Lys Phe Ile
            500                 505                 510
Thr Phe Ser Met Leu Ile Phe Phe Ile Val Trp Ile Ser Phe Ile Pro
        515                 520                 525
Ala Tyr Phe Ser Thr Tyr Gly Lys Phe Val Ser Ala Val Glu Val Ile
    530                 535                 540
Ala Ile Leu Ala Ser Ser Phe Gly Met Leu Ala Cys Ile Phe Phe Asn
545                 550                 555                 560
Lys Val Tyr Ile Ile Leu Phe Lys Pro Ser Arg Asn Thr Ile Glu Glu
                565                 570                 575
Val Arg Cys Ser Thr Ser Ala His Ala Phe Lys Val Ala Ala Lys Ala
            580                 585                 590
Thr Leu Lys His Ser Thr Ala Ser Arg Arg Lys Ser Gly Ser Thr Gly
        595                 600                 605
Gly Ser Ser Asp Ser Thr Pro Ser Ser Ser Ile Ser Leu Lys Thr Asn
    610                 615                 620
Gly Asn Asp Pro Thr Ser Gly Lys Pro Arg Val Ser Phe Gly Ser Gly
625                 630                 635                 640
Thr Val Thr Leu Ser Leu Ser Phe Glu Glu Ser Arg Arg Ser Ser Leu
                645                 650                 655
Met
```

FIG. 10C

tgtcgtggac ggagcccttt gggatcgcgt tggccatatg tgcagcgctg ggtgttgcct 60 SEQ ID NO. 21
tgacgggctt cgtgatggcc gtctttatca gattccgcaa caccccaata gtgaaggcca 120
cgaaccgaga actgtcctat gtcctcctgt tctctctcat ctgttgcttc tccagttccc 180
tcatctttat tggagagccg caggattgga tgtgtcgttt acgccaacct gcctttggga 240
tcagttttgt tctctgtatc tcctgcatcc ttgtgaaaac taatagagta ctcttagtat 300
ttgaagccaa gatccccaca agtctccatc gtaaatggtg ggggttaaac cttcagtttt 360
tgctggtgtt tctgtgcaca tttgtccaag tcatgatctg tgttgtctgg ctgtacaatg 420
cccctccctc cagttacagg aattatgaca tagatgagat gatttttatc acatg 475

Ser Trp Thr Glu Pro Phe Gly Ile Ala Leu Ala Ile Cys Ala Ala Leu SEQ ID NO. 22
1 5 10 15
Gly Val Ala Leu Thr Gly Phe Val Met Ala Val Phe Ile Arg Phe Arg
20 25 30
Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Val Leu
35 40 45
Leu Phe Ser Leu Ile Cys Cys Phe Ser Ser Ser Leu Ile Phe Ile Gly
50 55 60
Glu Pro Gln Asp Trp Met Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile
65 70 75 80
Ser Phe Val Leu Cys Ile Ser Cys Ile Leu Val Lys Thr Asn Arg Val
85 90 95
Leu Leu Val Phe Glu Ala Lys Ile Pro Thr Ser Leu His Arg Lys Trp
100 105 110
Trp Gly Leu Asn Leu Gln Phe Leu Leu Val Phe Leu Cys Thr Phe Val
115 120 125
Gln Val Met Ile Cys Val Val Trp Leu Tyr Asn Ala Pro Pro Ser Ser
130 135 140
Tyr Arg Asn Tyr Asp Ile Asp Glu Met Ile Phe Ile Thr
145 150 155

FIG. 11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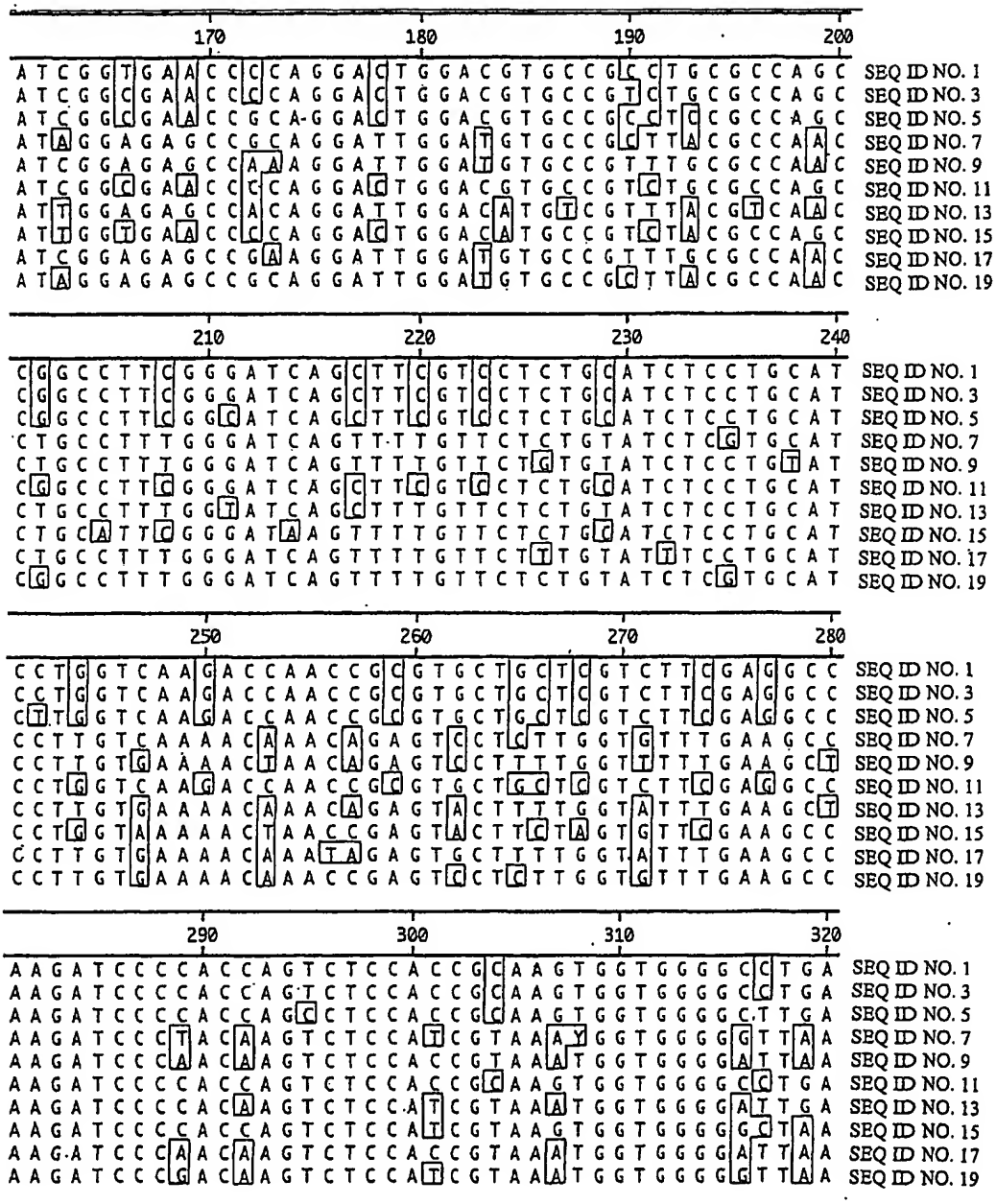
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19
50 60 70 80
SEQ ID NO. 1
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19
90 100 110 120
SEQ ID NO. 1
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19
130 140 150 160
SEQ ID NO. 1
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19

FIG. 12A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SEQ ID NO. 1
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19
210 220 230 240
SEQ ID NO. 1
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19
250 260 270 280
SEQ ID NO. 1
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19
290 300 310 320
SEQ ID NO. 1
SEQ ID NO. 3
SEQ ID NO. 5
SEQ ID NO. 7
SEQ ID NO. 9
SEQ ID NO. 11
SEQ ID NO. 13
SEQ ID NO. 15
SEQ ID NO. 17
SEQ ID NO. 19

FIG. 12B

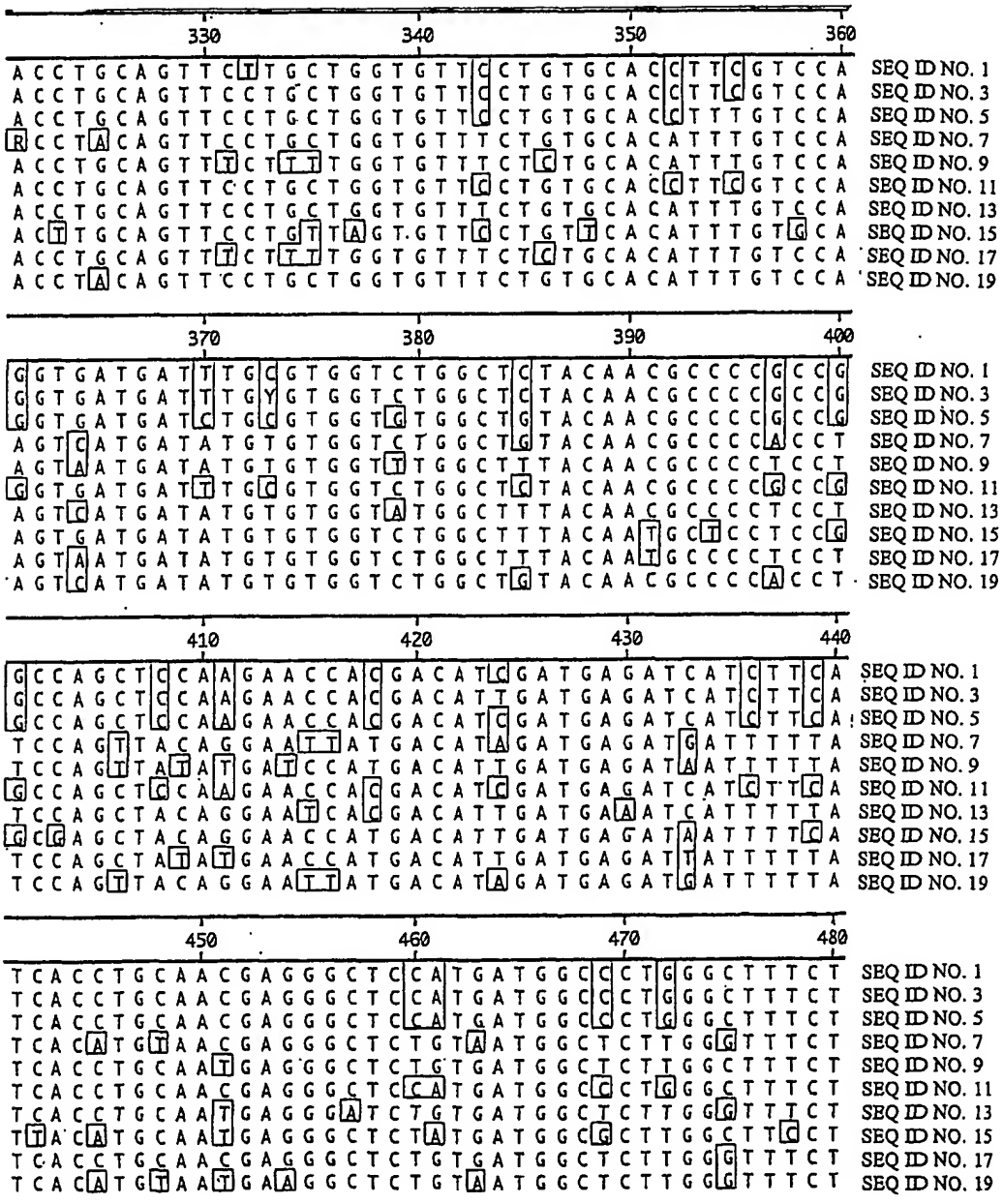
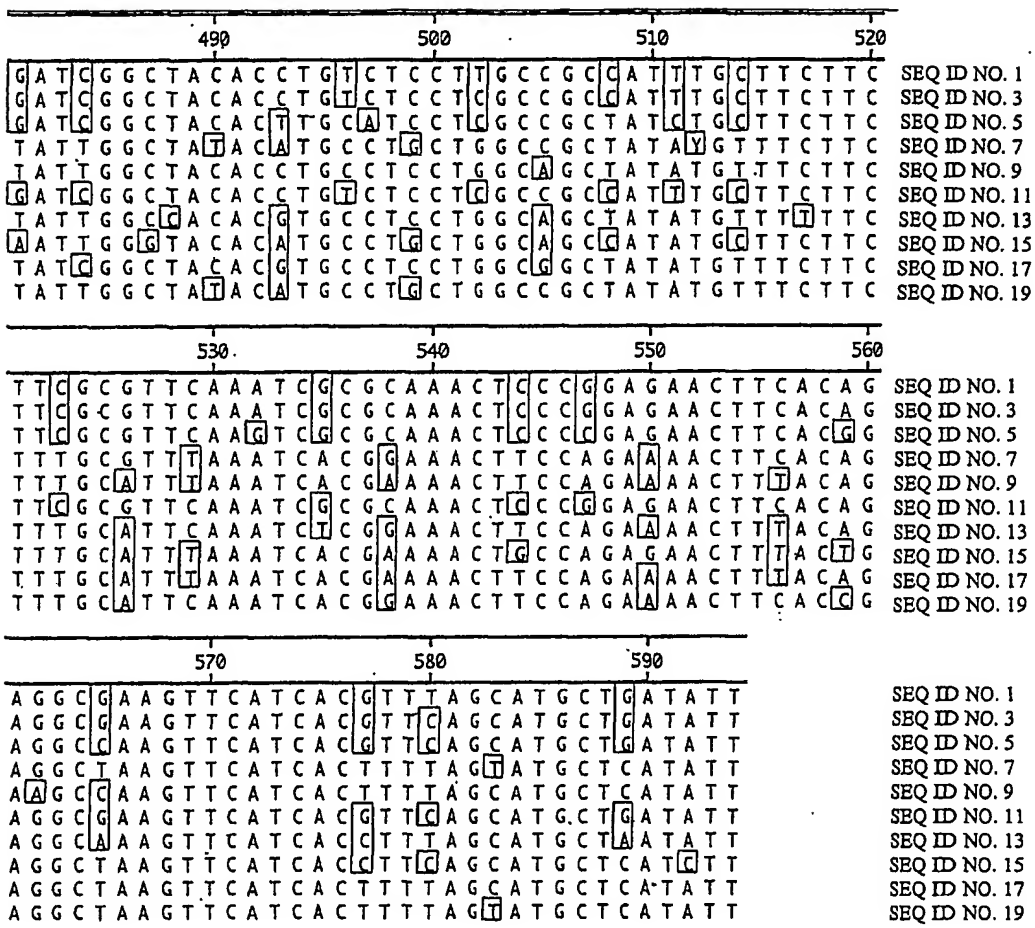

330 340 350 360
ACCTGCAGTTCTTGCTGGTGTTCCTGTGCACCTTCGTCCA SEQ ID NO. 1
ACCTGCAGTTCCTGCTGGTGTTCCTGTGCACCTTCGTCCA SEQ ID NO. 3
ACCTGCAGTTCCTGCTGGTGTTCCTGTGCACCTTTGTCCA SEQ ID NO. 5
RCCTACAGTTCCTGCTGGTGTTTCTGTGCACATTTGTCCA SEQ ID NO. 7
ACCTGCAGTTTCTTTGGTGTTTCTGCACATTTGTCCA SEQ ID NO. 9
ACCTGCAGTTCCTGCTGGTGTTCCTGTGCACCTTCGTCCA SEQ ID NO. 11
ACCTGCAGTTCCTGCTGGTGTTTCTGTGCACATTTGTCCA SEQ ID NO. 13
ACTTGCAGTTCCTGTTAGTGTTCCTGTTCACATTTGTGCA SEQ ID NO. 15
ACCTGCAGTTTCTTTGGTGTTTCTGCTGCACATTTGTCCA SEQ ID NO. 17
ACCTACAGTTCCTGCTGGTGTTTCTGTGCACATTTGTCCA SEQ ID NO. 19
370 380 390 400
GGTGATGATTTGCGTGGTCTGGCTCTACAACGCCCCGCCG SEQ ID NO. 1
GGTGATGATTTGYGTGGTCTGGCTCTACAACGCCCCGCCG SEQ ID NO. 3
GGTGATGATCTGCGTGGTGTGGCTGTACAACGCCCCGCCG SEQ ID NO. 5
AGTCATGATATGTGTGGTCTGGCTGTACAACGCCCCACCT SEQ ID NO. 7
AGTAATGATATGTGTGGTTTGGCTTTACAACGCCCCTCCT SEQ ID NO. 9
GGTGATGATTTGCGTGGTCTGGCTCTACAACGCCCCGCCG SEQ ID NO. 11
AGTCATGATATGTGTGGTATGGCTTTACAACGCCCCTCCT SEQ ID NO. 13
AGTGATGATATGTGTGGTCTGGCTTTACAATGCTCCTCCG SEQ ID NO. 15
AGTAATGATATGTGTGGTCTGGCTTTACAATGCCCCTCCT SEQ ID NO. 17
AGTCATGATATGTGTGGTCTGGCTGTACAACGCCCCACCT SEQ ID NO. 19
410 420 430 440
GCCAGCTCCAAGAACCACGACATCGATGAGATCATCTTCA SEQ ID NO. 1
GCCAGCTCCAAGAACCACGACATTGATGAGATCATCTTCA SEQ ID NO. 3
GCCAGCTCCAAGAACCACGACATCGATGAGATCATCTTCA SEQ ID NO. 5
TCCAGTTACAGGAATTATGACATAGATGAGATGATTTTTA SEQ ID NO. 7
TCCAGTTATTATGACATTGATGAGATAATTTTTA SEQ ID NO. 9
GCCAGCTCCAAGAACCACGACATCGATGAGATCATCTTCA SEQ ID NO. 11
TCCAGCTACAGGAATCACGACATTGATGAAATCATTTTTA SEQ ID NO. 13
GCGAGCTACAGGAACCATGACATTGATGAGATAATTTTCA SEQ ID NO. 15
TCCAGCTATATGAACCATGACATTGATGAGATTATTTTTA SEQ ID NO. 17
TCCAGTTACAGGAATTATGACATAGATGAGATGATTTTTA SEQ ID NO. 19
450 460 470 480
TCACCTGCAACGAGGGCTCCATGATGGCCCCTGGGCTTTCT SEQ ID NO. 1
TCACCTGCAACGAGGGCTCCATGATGGCCCCTGGGCTTTCT SEQ ID NO. 3
TCACCTGCAACGAGGGCTCCATGATGGCCCCTGGGCTTTCT SEQ ID NO. 5
TCACATGTAACGAGGGCTCTGTAATGGCTCTTGGGTTTCT SEQ ID NO. 7
TCACCTGCAATGAGGGCTCTGTGATGGCTCTTGGCTTTCT SEQ ID NO. 9
TCACCTGCAACGAGGGCTCCATGATGGCCCCTGGGCTTTCT SEQ ID NO. 11
TCACCTGCAATGAGGGATCTGTGATGGCTCTTGGGTTTCT SEQ ID NO. 13
TTACATGCAATGAGGGCTCTATGATGGCCGCTTGGCTTTCT SEQ ID NO. 15
TCACCTGCAACGAGGGCTCTGTGATGGCTCTTGGGTTTCT SEQ ID NO. 17
TCACATGTAATGAGGGCTCTGTAATGGCTCTTGGGTTTCT SEQ ID NO. 19

FIG. 12C



Decoration 'Decoration #1': Box residues that differ from the Consensus.

FIG. 12D

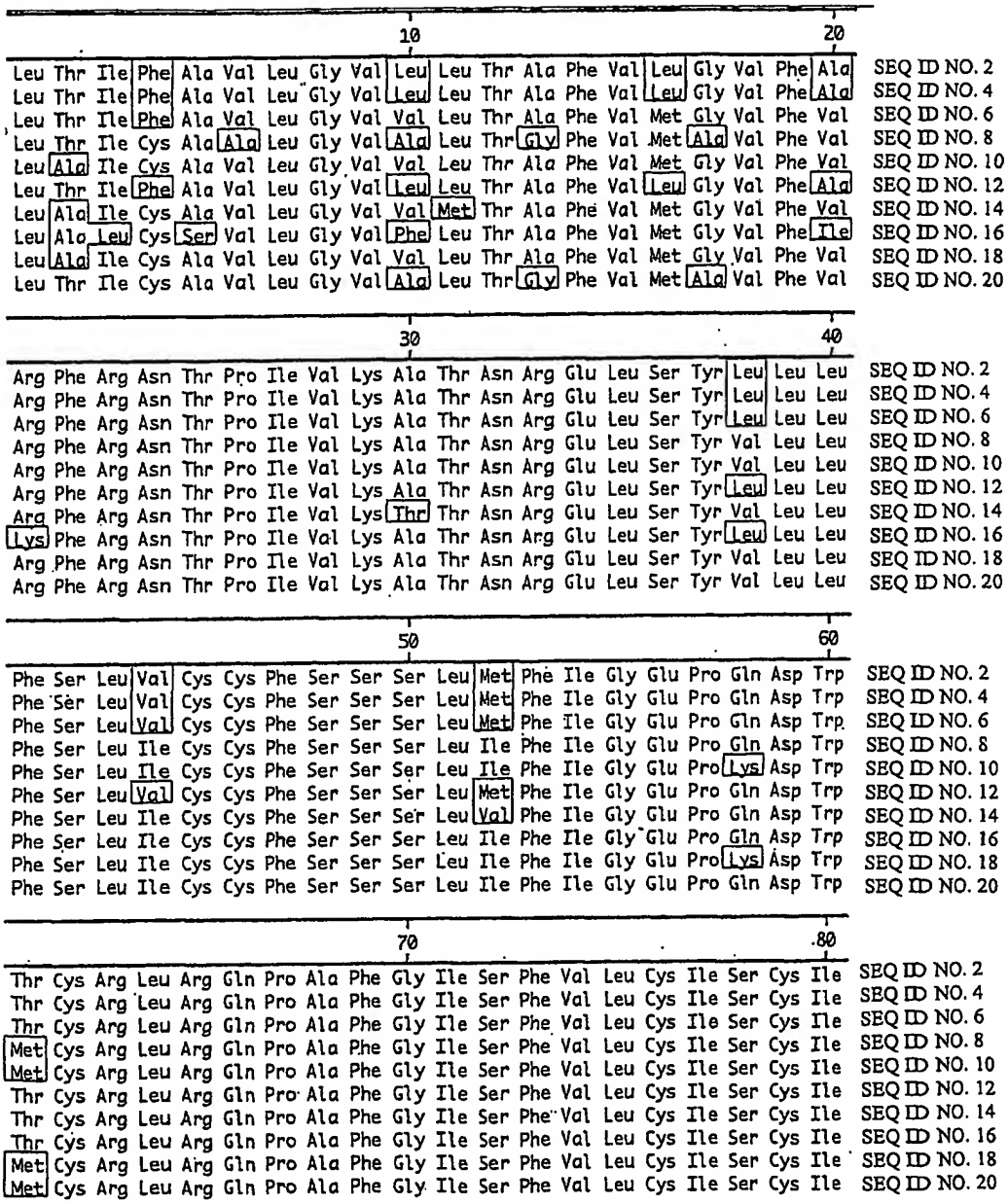

10 20
Leu Thr Ile Phe Ala Val Leu Gly Val Leu Leu Thr Ala Phe Val Leu Gly Val Phe Ala SEQ ID NO. 2
Leu Thr Ile Phe Ala Val Leu Gly Val Leu Leu Thr Ala Phe Val Leu Gly Val Phe Ala SEQ ID NO. 4
Leu Thr Ile Phe Ala Val Leu Gly Val Val Leu Thr Ala Phe Val Met Gly Val Phe Val SEQ ID NO. 6
Leu Thr Ile Cys Ala Ala Leu Gly Val Ala Leu Thr Gly Phe Val Met Ala Val Phe Val SEQ ID NO. 8
Leu Ala Ile Cys Ala Val Leu Gly Val Val Leu Thr Ala Phe Val Met Gly Val Phe Val SEQ ID NO. 10
Leu Thr Ile Phe Ala Val Leu Gly Val Leu Leu Thr Ala Phe Val Leu Gly Val Phe Ala SEQ ID NO. 12
Leu Ala Ile Cys Ala Val Leu Gly Val Val Met Thr Ala Phe Val Met Gly Val Phe Val SEQ ID NO. 14
Leu Ala Leu Cys Ser Val Leu Gly Val Phe Leu Thr Ala Phe Val Met Gly Val Phe Ile SEQ ID NO. 16
Leu Ala Ile Cys Ala Val Leu Gly Val Val Leu Thr Ala Phe Val Met Gly Val Phe Val SEQ ID NO. 18
Leu Thr Ile Cys Ala Val Leu Gly Val Ala Leu Thr Gly Phe Val Met Ala Val Phe Val SEQ ID NO. 20
30 40
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Leu Leu Leu SEQ ID NO. 2
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Leu Leu Leu SEQ ID NO. 4
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Leu Leu Leu SEQ ID NO. 6
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Val Leu Leu SEQ ID NO. 8
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Val Leu Leu SEQ ID NO. 10
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Leu Leu Leu SEQ ID NO. 12
Arg Phe Arg Asn Thr Pro Ile Val Lys Thr Thr Asn Arg Glu Leu Ser Tyr Val Leu Leu SEQ ID NO. 14
Lys Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Leu Leu Leu SEQ ID NO. 16
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Val Leu Leu SEQ ID NO. 18
Arg Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Val Leu Leu SEQ ID NO. 20
50 60
Phe Ser Leu Val Cys Cys Phe Ser Ser Ser Leu Met Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 2
Phe Ser Leu Val Cys Cys Phe Ser Ser Ser Leu Met Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 4
Phe Ser Leu Val Cys Cys Phe Ser Ser Ser Leu Met Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 6
Phe Ser Leu Ile Cys Cys Phe Ser Ser Ser Leu Ile Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 8
Phe Ser Leu Ile Cys Cys Phe Ser Ser Ser Leu Ile Phe Ile Gly Glu Pro Lys Asp Trp SEQ ID NO. 10
Phe Ser Leu Val Cys Cys Phe Ser Ser Ser Leu Met Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 12
Phe Ser Leu Ile Cys Cys Phe Ser Ser Ser Leu Val Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 14
Phe Ser Leu Ile Cys Cys Phe Ser Ser Ser Leu Ile Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 16
Phe Ser Leu Ile Cys Cys Phe Ser Ser Ser Leu Ile Phe Ile Gly Glu Pro Lys Asp Trp SEQ ID NO. 18
Phe Ser Leu Ile Cys Cys Phe Ser Ser Ser Leu Ile Phe Ile Gly Glu Pro Gln Asp Trp SEQ ID NO. 20
70 80
Thr Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 2
Thr Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 4
Thr Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 6
Met Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 8
Met Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 10
Thr Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 12
Thr Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 14
Thr Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 16
Met Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 18
Met Cys Arg Leu Arg Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile SEQ ID NO. 20

FIG. 13A

90														100	
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 2
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 4
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 6
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 8
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 10
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 12
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 14
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 16
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 18
Leu	Val	Lys	Thr	Asn	Arg	Val	Leu	Leu	Val	Phe	Glu	Ala	Lys	Ile	Pro	Thr	Ser	Leu	His	SEQ ID NO. 20

110														120	
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 2
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 4
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 6
Arg	Lys	Arg	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 8
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 10
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 12
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 14
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Phe	Thr	Phe	Val	Gln	SEQ ID NO. 16
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 18
Arg	Lys	Trp	Trp	Gly	Leu	Asn	Leu	Gln	Phe	Leu	Leu	Val	Phe	Leu	Cys	Thr	Phe	Val	Gln	SEQ ID NO. 20

130														140	
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ala	Ser	Ser	Lys	Asn	His	Asp	SEQ ID NO. 2
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ala	Ser	Ser	Lys	Asn	His	Asp	SEQ ID NO. 4
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ala	Ser	Ser	Lys	Asn	His	Asp	SEQ ID NO. 6
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ser	Ser	Tyr	Arg	Asn	Tyr	Asp	SEQ ID NO. 8
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ser	Ser	Tyr	Met	Ile	His	Asp	SEQ ID NO. 10
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ala	Ser	Ser	Lys	Asn	His	Asp	SEQ ID NO. 12
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ser	Ser	Tyr	Arg	Asn	His	Asp	SEQ ID NO. 14
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ala	Ser	Tyr	Arg	Asn	His	Asp	SEQ ID NO. 16
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ser	Ser	Tyr	Met	Asn	His	Asp	SEQ ID NO. 18
Val	Met	Ile	Cys	Val	Val	Trp	Leu	Tyr	Asn	Ala	Pro	Pro	Ser	Ser	Tyr	Arg	Asn	Tyr	Asp	SEQ ID NO. 20

150														160	
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Met	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 2
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Met	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 4
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Met	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 6
Ile	Asp	Glu	Met	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Val	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 8
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Val	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 10
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Met	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 12
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Val	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 14
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Met	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 16
Ile	Asp	Glu	Ile	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Val	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 18
Ile	Asp	Glu	Met	Ile	Phe	Ile	Thr	Cys	Asn	Glu	Gly	Ser	Val	Met	Ala	Leu	Gly	Phe	Leu	SEQ ID NO. 20

FIG. 13B

170														180	
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 2
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 4
Ile	Gly	Tyr	Thr	Cys	Ile	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 6
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Arg	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 8
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 10
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 12
Ile	Gly	His	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 14
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 16
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 18
Ile	Gly	Tyr	Thr	Cys	Leu	Leu	Ala	Ala	Ile	Cys	Phe	Phe	Phe	Ala	Phe	Lys	Ser	Arg	Lys	SEQ ID NO. 20

190													
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 2
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 4
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 6
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 8
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 10
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 12
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 14
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 16
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 18
Leu	Pro	Glu	Asn	Phe	Thr	Glu	Ala	Lys	Phe	Ile	Thr	Phe	Ser	Met	Leu	Ile	SEQ ID NO. 20

Decoration 'Decoration #1': Box residues that differ from the Consensus.

FIG. 13C

aattccgttg ctgtcggttc agtccaagtc tcctccagtg caaaatgaga aatggtggtc 60 SEQ ID NO. 23
gccattacag gaacatgcac tacatctgtg ttaatgaaat attgtcagtt atctgaaggt 120
tattaaaatg tttctgcaag gatggcttca cgagaaatca attctgcacg ttttcccatt 180
gtcattgtat gaataactga ccaaagggat gtaacaaaat ggaacaaagc tgaggaccac 240
gttcaccctt tcttggagca tacgatcaac cctgaaggag atggaagact tgaggaggaa 300
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gcagaaatcc tccaggcatc ctctgtaaac gggctggcgt agtgtggctt ggtcaaggaa 420
cagagacagg gctgcacaat ggctcagctt cactgccaac tcttattctt gggatttaca 480
ctcctacagt cgtacaatgt ctcagggtat ggtccaaacc aaagggccca gaagaaagga 540
gacatcatac tgggaggtct cttcccaata cactttggag tagccgccaa ggatcaggac 600
ttaaaatcga gaccggaggc gacaaaatgt attcggtaca attttcgagg cttccgatgg 660
ctccaggcga tgatattcgc aattgaagag attaacaaca gtatgacttt cctgcccaat 720
atcaccctgg gatatcgcat atttgacacg tgtaacaccg tgtccaaggc gctagaggca 780
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agcaggctgc tcagcaacaa gaatgagtac aaggccttcc tgaggaccat ccccaatgat 1020
gagcaacagg ccacggccat ggccgagatc atcgagcact tccagtggaa ctgggtggga 1080
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cggatctggc tggccagcga ggcttgggcc agctcttcgc tcattgccaa gccagagtac 1380
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ttctgggagg agaccttcaa ctgctacttc accgagaaga ccctgacgca gctgaagaat 1560
tccaaggtgc cctcgcacgg accggcggct caaggggacg gctccaaggc ggggaactcc 1620
agacggacag ccctacgcca cccctgcact ggggaggaga acatcaccag cgtggagacc 1680
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ggatcttgtg cagatattaa aaaagttgag gcctggcagg tcctcaacca tctgctgcat 1860
ctgaagttta ccaacagcat gggtgagcag gttgactttg acgatcaagg tgacctcaag 1920
gggaactaca ccattatcaa ctggcagctc tccgcagagg atgaatcggt gttgttccat 1980
gaggtgggca actacaacgc ctacgctaag cccagtgacc gactcaacat caacgaaaag 2040
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ctggtcttcc tctgcatcct ggtgcaaatc gtcacctgca tcatctggct ctacaccgcg 2700
cctccctcca gctacaggaa ccatgagctg gaggacgagg tcatcttcat cacctgcgac 2760
gagggctcgc tcatggcgct gggcttcctc atcggctaca cctgcctcct cgccgccatc 2820
tgcttcttct tcgccttcaa gtcccgtaag ctgccggaga acttcaacga ggctaagttc 2880
atcaccttca gcatgttgat cttcttcatc gtctggatct ccttcatccc cgcctatgtc 2940
agcacctacg gcaagtttgt gtcggccgtg gaggtgattg ccatcctggc ctccagcttc 3000
gggctgctgg gctgcattta cttcaacaag tgttacatca tcctgttcaa gccgtgccgt 3060
aacaccatcg aggaggtgcg ctgcagcacg gcggcccacg ccttcaaggt ggcggcccgg 3120
gccaccctcc ggcgcagcgc cgcgtctcgc aagcgctcca gcagcctgtg cggctccacc 3180
atctcctcgc ccgcctcgtc cacctgcggg ccgggcctca ccatggagat gcagcgctgc 3240
agcacgcaga aggtcagctt cggcagcggc accgtcaccc tgtcgctcag cttcgaggag 3300
acaggccgat acgccaccct cagccgcacg gcccgcagca ggaactcggc ggatggccgc 3360
agcggcgacg acctgccatc tagacaccac gaccagggcc cgcctcagaa atgcgagccc 3420
cagcccgcca acgatgcccg atacaaggcg gcgccgacca agggcaccct agagtcgccg 3480
ggcggcagca aggagcgccc cacaactatg gaggaaacct aatccaactc ctccatcaac 3540
cccaagaaca tcctccacgg cagcaccgtc gacaactgac atcaactcct aaccggtggc 3600
tgcccaacct ctccccctct cggcactttg cgttttgctg aagattgcag catctgcagt 3660

FIG. 14A

```
tccttttatc cctgattttc tgacttggat atttactagt gtgcgatgga atatcacaac 3720
ataatgagtt gcacaattag gtgagcagag ttgtgtcaaa gtatctgaac tatctgaagt 3780
atctgaacta ctttattctc tcgaattgta ttacaaacat ttgaagtatt tttagtgaca 3840
ttatgttcta acattgtcaa gataatttgt tacaacatat aaggtaccac ctgaagcagt 3900
gactgagatt gccactgtga tgacagaact gttttataac atttatcatt gaaacctgga 3960
ttgcaacagg aatataatga ctgtaacaaa aaaattgttg attatcttaa aaatgcaaat 4020
tgtaatcaga tgtgtaaaat tggtaattac ttctgtacat taaatgcata tttcttgata 4080
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaagcgg cccgacagca acgg       4134
```

FIG. 14B

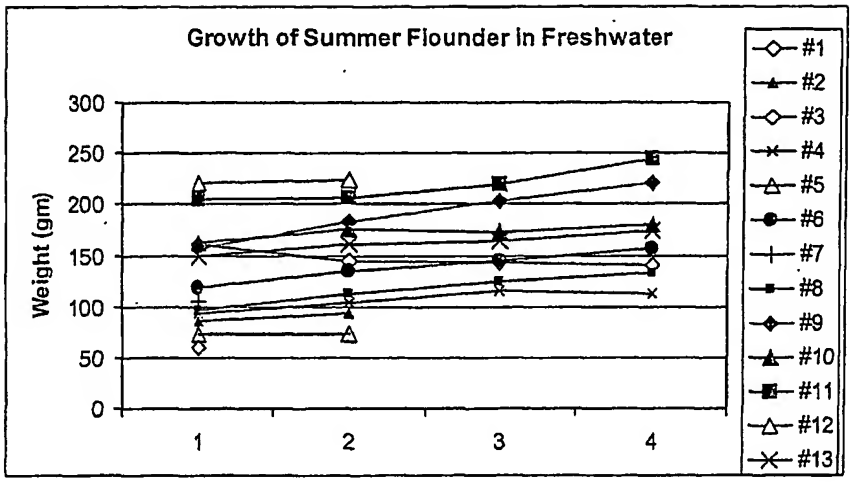
Growth of Summer Flounder in Freshwater
Weight (gm)
300
250
200
150
100
50
0
1
2
3
4
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13

FIG. 15

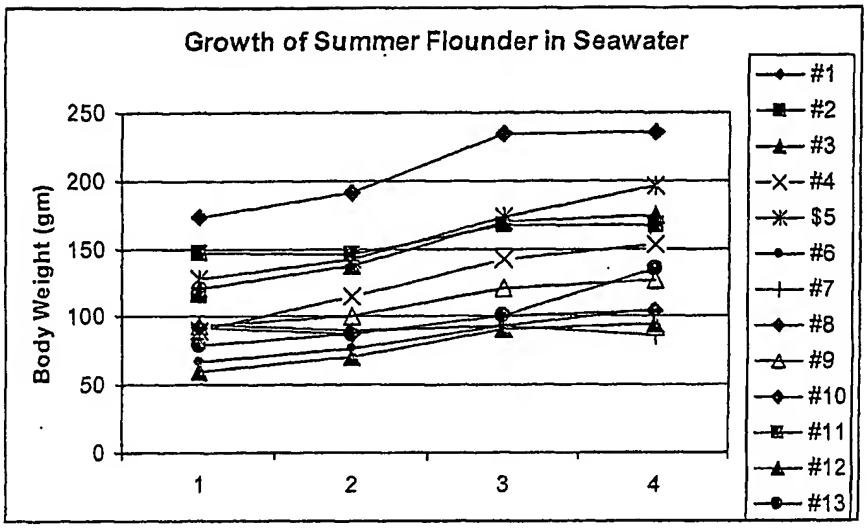
Growth of Summer Flounder in Seawater
Body Weight (gm)
250
200
150
100
50
0
1
2
3
4
#1
#2
#3
#4
$5
#6
#7
#8
#9
#10
#11
#12
#13

FIG. 16

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(72) Inventors; and

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(74) Agents: **BROOK, David, E.** et al.; Hamilton, Brook, Smith & Reynolds, P.C., 530 Virginia Road, P.O. Box 9133, Concord, MA 01742-9133 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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(54) Title: GROWING MARINE FISH IN FRESHWATER

(57) Abstract: The invention relates to methods, compositions and kits for raising marine fish in freshwater. The methods involve adding at least one Polyvalent Cation Sensing Receptor (PVCR) modulator to the freshwater in an amount sufficient to increase expression and/or sensitivity of at least one PVCR; and adding feed for fish consumption of the freshwater, wherein the feed comprises an amount of NaCl sufficient to contribute to a significant increased level of the PVCR modulator in serum of the marine fish.

INTERNATIONAL SEARCH REPORT

In ional Application No
PCT/US 01/31625

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23K1/18 A01K61/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 3 777 709 A (PHILLIPS G ET AL) 11 December 1973 (1973-12-11) claims	1,13,18, 19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2002	05/07/2002

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Ir ional Application No
PCT/US 01/31625

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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PCT/US 01/31625

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